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Microcosm Study of 1,4-Dioxane Biotransformation

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MICROCOSM STUDY OF 1,4-DIOXANE BIOTRANSFORMATION

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ABSTRACT

1,4-Dioxane is an emerging groundwater contaminant and probable human carcinogen with considerable potential remediation costs due to its hydrophilic and recalcitrant nature. Commonly occurring with 1,1,1-trichloroethane (1,1,1-TCA) plumes due to its application as a solvent stabilizer, 1,4-dioxane is also used in industrial lubricants and occurs as an impurity in numerous personal care products. Current treatment strategies to treat dioxane contamination include advanced oxidation processes and biological treatment, though only aerobic paths of biodegradation are currently understood. Laboratory and field studies have indicated the possible inhibitory effects of 1,1,1-TCA and its abiotically transformed daughter product, 1,1-dichloroethene (1,1-DCE) on the aerobic biodegradation of 1,4-dioxane.

The goal of this research was to evaluate the biodegradability of 1,4-dioxane in a variety of redox environments. The aerobic biodegradation of dioxane has been characterized in both laboratory and field studies, yet anaerobic processes related to this compound are poorly understood. The specific goals of this microcosm study were to evaluate: 1) anaerobic biodegradation of 1,4-dioxane in microcosms prepared with soil and groundwater amended with Fe(III), Fe(III)-ethylenediaminetetraacetic acid (EDTA), Fe(III)-EDTA + inoculum with samples from current microcosms that exhibit the highest level of 1,4-dioxane transformation, Fe(III) + anthraquinone-2,6-disulfonate (AQDS), and sulfate; 2) anaerobic biodegradation of 1,4-dioxane in microcosms prepared with soil and mineral salts medium amended with nitrate, Fe(III)-EDTA, and sulfate; 3) aerobic cometabolism of 1,4-dioxane by indigenous propanotrophs and by bioaugmentation with

a mixed culture of propanotrophs; 4) the effect of 1,1-DCE on aerobic cometabolism of 1,4-dioxane by propanotrophs; 5) the potential for sequential anaerobic dechlorination of 1,1-DCE to ethene followed by aerobic cometabolic biodegradation with propanotrophs; and 6) the presence of indigenous aerobic microbes capable of biodegrading 1,4-dioxane as their sole source of carbon and energy.

The results of this research showed that: 1) 1,4-dioxane is recalcitrant under all anaerobic environment in which it acts as the sole electron donor; 2) the addition of readily degradable substrates may stimulate the biodegradation of 1,4-dioxane in sulfate, nitrate, and ferric iron reducing conditions, although the only ferric iron amended bottles that showed the possibility of 1,4-dioxane disappearance also contained a humic acid analog; 3) 1,4-dioxane is readily degraded to concentrations below 25 µg/L in aerobic environments in the presence of propanotrophs; 4) the mixed propanotroph culture, ENV487, is greatly inhibited by the presence of low concentrations of 1,1-DCE; 5) utilizing reductive dechlorinating bacteria to biodegrade 1,1-DCE to ethane prior to the establishment of aerobic conditions allows the cometabolic activity of ENV487 to proceed uninhibited, and; 6) bacteria capable of utilizing 1,4-dioxane as a sole source of energy and carbon occur in aerobic environments and are readily grown in a laboratory setting.

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List of Abbreviations

ACGIH	American Conference of Governmental Industrial Hygienists
AQDS	anthraquinone 2,6-disulfonate
AMS	ammonia mineral salts
BSM	basal salts medium
COD	chemical oxygen demand
1,1-DCE	1,1-dichloroethene
DCM	dichloromethane
DDI	distilled de-ionized
DHHS	Department of Health and Human Services
EDTA	ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
GC	gas chromatograph
HPLC	high performance liquid chromatography
MSM	mineral salts medium
NIOSH	National Institute for Occupational Safety and Health
1,1,1-TCA	1,1,1-trichloroethane
VC	vinyl chloride
VOCs	volatile organic compounds

CHAPTER ONE

INTRODUCTION

1.1 Overview

1,4-Dioxane is a synthetic organic compound commonly used as a stabilizer for various chlorinated solvents. The structure consists of a six member diether ring, which often occupies one of two stable chair conformations but may also shift between two other boat conformations (Mohr, 2010). 1,4-Dioxane is used in various industrial processes, including a stabilizer for chlorinated solvents, most notably 1,1,1-trichloroethane (1,1,1-TCA), production of cellulose acetate membrane filters, and wetting and dispersing in the textile industry (EPA, 2006). Products that contain 1,4-dioxane include paint strippers, dyes, greases, and waxes. Consumer products such as deodorants, shampoos, and cosmetics may contain trace amounts of 1,4-dioxane as an impurity (EPA, 2006).

1,4-Dioxane is considered an emerging contaminant by the Environmental Protection Agency (EPA) due to its anticipated carcinogenicity to humans through all routes of exposure. The United States Department of Health and Human Services (DHHS), American Conference of Governmental Industrial Hygienists (ACGIH), and National Institute for Occupational Safety and Health (NIOSH) have also issued statements pertaining to the potential risks attributed to 1,4-dioxane exposure (EPA, 2006). Although no federal regulations have been implemented regarding 1,4-dioxane, several states have implemented reporting or guideline concentrations at or below 1 μg/L (EPA, 2006).

Environmental 1,4-dioxane contamination is typically associated with 1,1,1-TCA plumes, though due to abiotic transformation via dehydrohalogenation, 1,1-dichloroethene (1,1-DCE) is also a common co-contaminant associated with 1,4-dioxane (EPA, 2006). While these chlorinated solvent plumes are typically well delineated, relatively few of these sites have been analyzed for 1,4-dioxane despite the frequency at which both chlorinated solvents and 1,4-dioxane coincide at sites analyzed for both (Adamson et. al., 2014).

1,4-Dioxane is considered to be recalcitrant in the environment due to the stability of its chemical structure, which remains intact unless in the presence of high concentrations of acids, strong oxidizing agents, and high temperatures and pressures (Mohr, 2010). The properties of 1,4-dioxane related to its symmetrical ether linkages result in a compound that is highly miscible in water and sorbs poorly to organics (Mohr, 2010). This high affinity for water and low sorption to soils has led to the belief that 1,4-dioxane plumes are longer and more dilute than co-occurring chlorinated solvent plumes, though recent work has shown that for a majority of sites with available 1,4-dioxane data, the opposite is true (Adamson et. al., 2014).

Despite the recalcitrant nature of 1,4-dioxane, evidence of substantial aerobic biodegradation has been reported in both laboratory and field studies. A positive correlation has been established between environmental 1,4-dioxane attenuation and dissolved oxygen concentrations (Adamson et. al., 2015). Several types of bacteria metabolically degrade 1,4-dioxane, the most well studied example being *Pseudonocardia dioxanivorans* strain CB1190. Other bacteria are capable of co-metabolically degrading

1,4-dioxane in the presence of a primary substrate that induces the expression of oxygenases. These substrates include tetrahydrofuran (THF), short chain aliphatic compounds (including methane and propane), and toluene (Lippincott et al., 2015). Propane is advantageous because it is readily available, non-toxic, convenient to use, relatively inexpensive, dissolves adequately in water, and many soils contain indigenous propanotrophs. The ability to utilize 1,4-dioxane as a growth substrate is dependent on the bacteria's ability to mineralize the metabolites formed from the initial oxygenase attack of the ring structure (Mahendra et al., 2007).

While aerobic cometabolic bioremediation of 1,4-dioxane has reportedly been successful *in situ* via bioaugmentation with propanotrophs (Lippincott et. al., 2015), the chlorinated solvents that commonly co-occur with 1,4-dioxane present challenges to this strategy. A negative correlation has been established between 1,4-dioxane attenuation and chlorinated solvent concentrations, suggesting that these co-contaminants may be inhibitory to 1,4-dioxane attenuation (Adamson et. al., 2015). Laboratory studies have also shown that the aerobic degradation of 1,4-dioxane by both metabolic and co-metabolic degraders is inhibited by the presence of 1,1,1-TCA and 1,1-DCE (Mahendra et. al., 2013). While the inhibitory effects of these compounds were reversible in the 1,4-dioxane metabolizing strain CB1190, the co-metabolic bacteria degradation rates did not recover following the removal of the chlorinated solvents, suggesting an irreversible inhibitory effect on these microbes (Mahendra et. al., 2013).

Although progress with biodegradation of 1,4-dioxane under aerobic conditions has been promising, creating aerobic conditions *in situ* poses other significant challenges.

Development of anaerobic processes for remediating 1,4-dioxane is needed. Anaerobic 1,4-dioxane biodegradation has been reported in anaerobic digester sludge amended with Fe(III) (Shen et. al., 2008). The presence of humic acids to these enrichments further stimulated the activity. Up to 90% of the 1,4-dioxane was degraded with reportedly more than 50% of the carbon from the 1,4-dioxane mineralized to CO_2 (Shen et. al., 2008). However, this assertion was based on measurement of headspace CO_2; ^{14}C-labeled 1,4-dioxane was not used.

The Freedman laboratory at Clemson University has been evaluating anaerobic biodegradation of 1,4-dioxane with samples from two industrial sites. Field data from both suggest that 1,4-dioxane was undergoing natural attenuation in groundwater under anaerobic conditions. The concentration of 1,4-dioxane in these source areas was approximately 13-18 mg/L. Acetone and isopropyl alcohol were present at exceptionally high concentrations, with combined levels of 12,500 mg/L at one of the sites and 1,970 mg/L at the other. The concentration of each of these compounds decreased significantly downgradient in a short distance. A comparison to the decrease in halogenated organic compounds (i.e., 1,1-DCE at one site and Freons at the other) suggests that the decrease in the non-chlorinated compounds was due to biodegradation rather than simple dilution. Microcosms were prepared with soil and groundwater from both sites, with amendments that included Fe(III), Fe(III)-EDTA, Fe(III) + anthraquinone-2,6-disulfonate (AQDS), and sulfate. After more than three years of incubation, there was no definitive evidence for anaerobic biodegradation of 1,4-dioxane. Given the apparent attenuation of 1,4-dioxane based on field monitoring data, the lack of anaerobic biodegradation of 1,4-

dioxane in the microcosms was puzzling. These results indicated that additional efforts are needed to evaluate the potential for anaerobic biodegradation of 1,4-dioxane in contaminated aquifers.

The subject of this thesis is an evaluation of 1,4-dioxane at two additional industrial sites, hereafter referred to as Sites I and II. At Site I, 1,4-dioxane is present along with 1,1-DCE. At Site II, 1,4-dioxane is the only contaminant of concern. Microcosms were prepared with soil and groundwater from both sites in order to evaluate anaerobic biodegradation of 1,4-dioxane. Aerobic biodegradation was also evaluated, including treatments to evaluate propanotrophic cometabolism.

1.2 Objectives

The overall objective of this research was to evaluate the potential for natural and enhanced biodegradation of 1,4-dioxane at industrial Sites I and II, under anaerobic and aerobic conditions. The specific objectives were to evaluate:

1) anaerobic biodegradation of 1,4-dioxane in microcosms prepared with soil and groundwater amended with Fe(III), Fe(III)-EDTA, Fe(III)-EDTA + inoculum with samples from current microcosms that exhibit the highest level of 1,4-dioxane transformation, and Fe(III) + AQDS;

2) anaerobic biodegradation of 1,4-dioxane in microcosms prepared with soil and mineral salts medium amended with nitrate, Fe(III)-EDTA, and sulfate;

3) aerobic cometabolism of 1,4-dioxane by indigenous propanotrophs and by bioaugmentation with a mixed culture of propanotrophs;

4) the effect of 1,1-DCE on aerobic cometabolism of 1,4-dioxane by propanotrophs;

5) the potential for sequential anaerobic dechlorination of 1,1-DCE to ethene followed by aerobic cometabolic biodegradation with propanotrophs; and

6) the presence of indigenous aerobic microbes capable of biodegrading 1,4-dioxane as their sole source of carbon and energy.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Chemicals and Mineral Salts Medium

The sources and purity of chemicals used are summarized in Table 2.1. Chemicals that are not listed were reagent grade or an equivalent. An Fe(III)-gel consisting of amorphous Fe(III) oxyhydroxide was prepared as described by Lovely and Philips (1986). The compositon of an anaerobic mineral salts medium (MSM) that was used is described by Adrian et al. (1998) and Tschech and Pfeffig (1984); the procedure used to prepare the MSM is in Appendix A. The composition of an aerobic basal salt medium (BSM) is described by Hareland et al. (1975); the procedure used to prepare the BSM is in Appendix B. The composition of an ammonium mineral salt (AMS) medium is described by Parales (1994); the procedure used to prepare the ASM medium is in Appendix C.

2.2 Experimental Plan for Microcosms

Microcosms were prepared using soil and groundwater from industrial Sites I and II. Site I is in California and the contaminants include 1,4-dioxane and 1,1-DCE. Soil cores were collected 8.5-9.1 m, which was below the water table. Site II is in South Carolina and 1,4-dioxane the only contaminant of concern. The depth to the groundwater is less than 5 m.

Eight treatments were prepared, in triplicate:

- Unamended

- Addition of ferric iron gel
- Addition of chelated ferric iron
- Addition of chelated ferric iron + inoculum with samples from current microcosms that exhibit the highest level of 1,4-dioxane transformation
- Addition of ferric iron + anthraquinone 2,6-disulfonate (AQDS)
- Addition of sulfate
- Killed controls
- Water controls

The unamended treatment was designed to simulate *in situ* conditions. Addition of ferric iron, chelated ferric iron, AQDS (an analogue of humic acids), and sulfate was intended to determine if 1,4-dioxane transformation was associated with these electron acceptors.

Killed controls were used to determine the extent of 1,4-dioxane loss due to abiotic processes, while water controls indicated the extent of losses solely by diffusion through the serum bottle septa.

Microcosms were prepared with soil and groundwater samples taken from both sites. For Site I, the first set of samples was obtained on September 26, 2013 and shipped overnight on ice to Clemson University. These microcosms are designated Site I Set I.

Based on concerns that the Set I soil sample was not handled properly (i.e., it arrived in plastic bags rather than as cores), a new set of samples was requested. The second set of samples was taken on January 20, 2014 and shipped overnight on ice to

Clemson University. Microcosms prepared with these samples are designated Site I Set II.

For Site II, the soil and groundwater samples were obtained on April 2, 2014 and shipped overnight on ice to Clemson University. Since only one set of microcosms was prepared, there is no designation of the set number as with the Site I microcosms.

Using soil from Site I Set II and Site II, a set of anaerobic microcosms was also prepared with MSM in place of groundwater. The intent was to explore if anaerobic biodegradation might be limited by the absence of a nutrient present in the medium that is lacking in the soil or groundwater. The following treatments were prepared:

- Unamended
- Addition of Fe(III)-EDTA
- Addition of nitrate
- Addition of sulfate

The amounts of Fe(III)-EDTA, nitrate and sulfate added were based on the amount of electron acceptor required for full oxidation of the initial 1,4-dioxane concentrations (10 mg/L). A safety factor of 8 was applied to both Site I and Site II sets of soil + groundwater microcosms. A safety factor of 1.5 was used to calculate the amount of electron acceptor added to the soil + MSM microcosms.

Aerobic biodegradation of 1,4-dioxane was also evaluated. The following treatments were prepared, in triplicate:

- No amendments other than ensuring oxygen was present
- Biostimulation with propane and oxygen

- Bioaugmentation with ENV487, propane, and oxygen

ENV487 is a mixed culture of propanotrophs obtained courtesy of Dr. Robert Stefan at Chicago Bridge and Iron, Inc.

2.3 Microcosm Preparation and Monitoring

2.3.1 Anaerobic Microcosms with Soil + Groundwater

For Site I, samples of groundwater were received from four wells (Table 2.2). The 1,4-dioxane concentration was measured in each sample prior to preparing the microcosms, for both Sets I and II. As shown in Table 2.2, the initial concentrations were below 1.5 mg/L. The contribution of 1,4-dioxane from the soil was determined by combining 20 g of soil with 50 mL of distilled de-ionized (DDI) water, incubating for approximately 1 d, and then sampling the water. For Sets I and II, the contribution of 1,4-dioxane from the soil was negligible. Groundwater from the different wells was composited (total of 2 L) and resazurin was added (1 mg/L). To facilitate detection of biodegradation activity, the initial concentration of 1,4-dioxane in the groundwater was increased to approximately 10 mg/L (using a 20 g/L stock solution of 1,4-dioxane). The composited sample of groundwater was then placed in the anaerobic chamber.

For Site II, the initial concentration of 1,4-dioxane in the groundwater was above 10 mg/L, so it was unnecessary to add any. The groundwater was obtained from a single well; 400 mL from each sample bottle was composited and resazurin was added. The groundwater was then placed in the anaerobic chamber.

For Site I Set I, the soil was received in plastic bags. The bags were transferred to the anaerobic chamber; the soil was emptied into a sterile plastic container and then

homogenized by mixing. For Site I Set II and Site II, soil cores were transferred to the anaerobic chamber; the soil was dislodged from the core into a sterile plastic container and then homogenized by mixing. Serum bottles (160 mL) were also transferred to the anaerobic chamber.

For treatments amended with Fe(III)-EDTA and AQDS, these compounds were added to the serum bottles first, as neat compounds. Once the serum bottles were in the anaerobic chamber, 20 g of composited soil and 50 mL of composited groundwater was added. The bottles were temporarily capped with slotted gray butyl rubber septa, removed from the anaerobic chamber, and the headspace was sparged with high purity N_2 for approximately 1 min to remove H_2 that was present within the chamber. The bottles were then sealed with slotted gray butyl rubber septa and aluminum crimp caps.

The initial amount of electron acceptor provided was in excess of the amount needed to satisfy complete oxidation of the 1,4-dioxane: 0.9 mL of an Fe(III)-gel containing 1.0 M Fe(III) (added in the anaerobic chamber); 0.32 g Fe(III)-EDTA; 0.18 g AQDS; 0.5 mL of 0.22 M sodium sulfate (added in the anaerobic chamber).

For Site I Set II and Site II, one of the treatments was bioaugmented with the homogenized contents of microcosms from a different site that is also contaminated with 1,4-dioxane. The microcosms had been amended with Fe(III)-EDTA and incubated for ~1,100 days before removing samples (~1 mL) and compositing the samples in the anaerobic chamber. Aliquots (0.5 mL) of the composited material was injected into the three bioaugmented bottles for Site I Set II and the three for Site II.

Killed controls were prepared by autoclaving triplicate microcosms for one hour on three consecutive days. After the third autoclaving, glutaraldehyde (50% w/w, Fisher Scientific) was added (14 g/L) to further reduce the potential for biotic activity (Rothermich, 2002). Water controls were prepared with autoclaved DDI water and sufficient amounts of 1,4-dioxane to yield initial concentrations that were similar to the live microcosms.

For the Site I Set I and Set II anaerobic microcosms, 2 µL of a ^{14}C-1,4-dioxane stock solution was added after the bottles were constructed. Samples of the liquid were then removed to determine total ^{14}C activity by direct addition to liquid scintillation cocktail. Additional samples were filtered (0.20 µm PTFE) and the filtrate was injected onto an HPLC to quantify the percentage of activity that was associated with 1,4-dioxane.

2.3.2 Anaerobic Microcosms with Soil + MSM

Anaerobic microcosms with MSM were prepared with soil from Site I Set II and Site II. The serum bottles and MSM were placed in the anaerobic chamber, where the homogenized soil was stored at room temperature in a sealed plastic container. The soil and MSM were dispensed to the serum bottles. The bottles were temporarily capped with slotted gray butyl rubber septa, removed from the anaerobic chamber, and the headspace was sparged with high purity N_2 for approximately 1 min to remove H_2 that was present within the chamber. The bottles were then sealed with slotted gray butyl rubber septa and aluminum crimp caps. Samples were removed to determine the background concentration of 1,4-dioxane, which was below 1.5 mg/L. The initial concentration of 1,4-dioxane was increased to approximately 10 mg/L.

The initial amount of electron acceptor provided was sufficient to satisfy complete oxidation of the 1,4-dioxane, plus a 50% excess: 0.063 g Fe(III)-EDTA (added in the anaerobic chamber, prior to adding the soil and MSM); 0.5 mL of 0.043 M sodium sulfate (added in the anaerobic chamber); and 0.05 mL of 0.068 M sodium nitrate (added in the anaerobic chamber).

2.3.3 Aerobic Microcosms with Soil + Groundwater

Aerobic microcosms were prepared with soil and groundwater samples from Site I Set II and Site II. Because the soil was being stored in the anaerobic chamber, the bottles were prepared inside by adding 20 g of homogenized soil and 50 mL of composited groundwater (with resazurin added). The bottles were temporarily capped with slotted gray butyl rubber septa, removed from the anaerobic chamber, and the headspace was sparged with room air for approximately 5 min to establish aerobic conditions. The bottles were then sealed with slotted gray butyl rubber septa and aluminum crimp caps. Samples were removed to determine the background concentration of 1,4-dioxane. For the Site I Set II bottles, the initial concentration was below 1.5 mg/L; consequently, 1,4-dioxane was added to yield a concentration of approximately 10 mg/L. For the Site II microcosms, the initial concentration was approximately 17 mg/L, so none was added. The initial amount of propane added was 2.0 mL, equivalent to approximately 82 µmol. When taking into account partitioning between the headspace (99 mL) and liquid (50 mL; the soil occupied 11 mL) using a dimensionless Henry's Law constant of 28.9 at 25°C, the initial aqueous phase concentration was 1.1 mg/L (see section 2.6.4 for converting mass per bottle to an

aqueous phase concentration). Barajas (personal communication) determined a transformation yield (T_y) for ENV487 of 0.59 mg 1,4-dioxane/mg propane. The initial ratio of 1,4-dioxane to propane in the microcosms was 0.14, indicating propane was in excess of the amount needed.

When the oxygen level in the headspace fell below 5%, the headspace of the bottles was sparged with room air, restoring the oxygen to 21%. ENV487 was added after growing it in BSM, with propane serving as the growth substrate. The biomass concentration reached approximately 650 mg protein/L; 0.5 mL was added to the microcosms, resulting in an initial ENV487 concentration of 6.5 mg protein/L. All of the microcosms were incubated quiescently at room temperature (22-24°C).

2.4 Effect of 1,1-DCE

2.4.1 Bioaugmented Aerobic Microcosms with Soil + Groundwater

The effect of 1,1-DCE on biodegradation of propane and 1,4-dioxane was evaluated in the bioaugmented treatment for the aerobic Site I microcosms. 1,1-DCE was added using a water-saturated solution (~2.25 mg/mL) to achieve an aqueous concentration of 1 mg/L, based on a Henry's Law constant of 0.971 at 23°C. The septa were changed to Teflon-faced gray butyl rubber septa, to minimize diffusive losses of 1,1-DCE through the septa.

2.4.2 Sequential Anaerobic 1,1-DCE and Aerobic 1,4-Dioxane Biodegradation

As the results will show, 1,1-DCE strongly inhibited ENV487 co-metabolic activity at concentrations above 0.1 mg/L. Consequently, a sequential anaerobic/aerobic approach was evaluated. An anaerobic reductive dechlorinating enrichment culture

(MicroCED) was employed to reductively dechlorinate 1,1-DCE to ethene, followed by a switch to aerobic cometabolism of 1,4-dioxane by ENV487. The unamended aerobic Site I Set II microcosms were used for this experiment. The serum bottles were transferred to the anaerobic chamber, uncapped, and allowed to equilibrate with the anaerobic atmosphere for 6 h. They were then recapped with Teflon faced butyl rubber septa, and 1,1-DCE was added as a saturated water solution to achieve an aqueous phase concentration of 1.0 mg/L. MicroCED, a bioaugmentation enrichment culture that grows by halorespiration of chlorinated aliphatic compounds (Yu et al., 2013), was added (0.5 mL) as well as lactate (70 µL of a stock solution containing 242 g/L of 60% sodium lactate syrup) to serve as electron donor. Sufficient lactate was added to account for reduction of the sulfate in the Site I groundwater (83.2 mg/L) and reduction of 1,1-DCE to ethene. When reductive dechlorination concluded, the microcosms were transferred out of the anaerobic chamber and sparged with room air passed through a sterile filter (0.2 µm, PTFE) for 3 min to establish aerobic conditions. ENV487 and propane were added to achieve concentrations of 6.5 mg protein/L and 1.1 mg aqueous propane/L, respectively.

2.4.3 Inhibition of ENV487 by 1,1-DCE

Aerobic incubations were prepared to further investigate the inhibitory effects of 1,1-DCE on co-metabolic degradation of 1,4-dioxane by ENV487. BSM (50 mL) and glass beads (sufficient to displace 11 mL, or the volume equivalent to 20 g soil) were added to each serum bottle (160 mL). Three concentrations of 1,4-dioxane (10, 30, and 50 mg/L) were evaluated. The following treatments were prepared in triplicate for each concentration:

- Addition of propane
- Addition of propane + 0.01 mg/L 1,1-DCE (aqueous)
- Addition of propane + 0.10 mg/L 1,1-DCE (aqueous)
- Addition of propane + 1.0 mg/L 1,1-DCE (aqueous)

To investigate the inhibitory effects of 1,1-DCE on only propane consumption, the following treatments were prepared in triplicate with no 1,4-dioxane added:

- Addition of propane + 0.01 mg/L 1,1-DCE (aqueous)
- Addition of propane + 0.10 mg/L 1,1-DCE (aqueous)
- Addition of propane + 1.0 mg/L 1,1-DCE (aqueous)

A sterile control containing 10 mg/L of 1,4-dioxane and 1.0 mg/L 1,1-DCE (aqueous) was prepared in triplicate to account for any abiotic losses of these compounds.

ENV487 was added to each bottle at the same initial concentration as previous aerobic microcosms (6.5 mg protein/L). The amount of propane added to each bottle was also the same as the previous aerobic microcosms (1.1 mg/L). The bottles were stored quiescently at room temperature (22-24°C).

2.5 Aerobic Enrichment of 1,4-Dioxane Metabolizing Bacteria

Results from the unamended aerobic Site II microcosms suggested the presence of bacteria capable of utilizing 1,4-dioxane as a growth substrate. Enrichments were prepared, in triplicate, in 160 mL serum bottles with 99 mL of AMS medium and 20 mg/L of 1,4-dioxane. The bottles were sealed with butyl rubber stoppers and capped with aluminum crimps. An aliquot (1 mL) of slurry was withdrawn from each microcosm and composited. An aliquot (1 mL) of this composite was added to each enrichment bottle.

Bottles were stored at room temperature on a shaker table (100 rpm) to increase the rate of aqueous phase oxygen transfer. The concentration of 1,4-dioxane was monitored, as well as oxygen levels in the headspace. When oxygen levels dropped below 5%, the bottles were sparged with room air passed through a sterile filter (0.2 µm) for 5 min to re-establish atmospheric levels.

2.6 Analytical Methods

2.6.1 1,4-Dioxane

1,4-Dioxane was monitored by gas chromatographic (GC) analysis of aqueous samples. Samples (1.0 mL) of the liquid phase were withdrawn from microcosms after allowing the solids to settle out. The water was passed through a syringe filter (0.2 µm PTFE, 13 mm diameter; VWR); the first 0.65 mL was discarded and the balance was discharged into a GC vial (1.8 mL Kimble ROBO Vial™) with a 400 µL borosilicate glass insert (VWR) and then capped (PTFE/red silicone septum, Agilent Technologies). Filtered samples (1.0 µL) were injected in splitless mode onto a Hewlett Packard 5890 Series II Plus GC, equipped with a flame ionization detector and a 60-m x 0.32-mm ZB-624 capillary column (Phenomenex). The temperature program was 60 °C for 5 min, then increased to 80 °C at a rate of 3.6 °C/min and held for 1 min, for a total run time of 11.6 min. The injector and detector temperatures were 180 °C and 260 °C, respectively. The carrier gas (H_2) flow rate was 1.2 mL/min. 1,4-Dioxane eluted at 6.8 min. A typical standard curve is shown in Figure 2.1a. The lowest standard used was 1.0 mg/L. However, concentrations as low as 400 µg/L were detectable, on the basis of peak areas that were two times greater than the background noise.

Standard additions were used to evaluate matrix effects. Percent recoveries were determined according to equation 2.1:

$$\%Recovery = \frac{B - 0.5A}{C} \times 100 \quad (2.1)$$

where A = sample; B = equal volumes of sample + standard; and C = equal volumes of the standard and DDI water. Based on 18 samples evaluated, the average percent recovery was 95.8±14.6, indicating no significant matrix interferences.

To achieve a lower quantifiable concentration, an alternate sample preparation method was used. Micro-frozen extractions of aqueous samples using dichloromethane (DCM) were prepared by adding a 3.0 mL of filtered aqueous samples to a 4 mL glass vial in which 0.6 mL of DCM was also added. This resulted in a volumetric sample to DCM ratio of 5.0. The mixture was then capped with a screw-on lid equipped with a rubber septum. The vials were agitated manually for 15 s and then vortexed for 15 s to ensure adequate mixing of both liquid phases. The vials were then placed upside down in a glass beaker, to allow the DCM phase to be in contact with the screw cap. The beakers with the vials were placed in a freezer (-20 °C) at a 45° angle for 1 hour. After the aqueous phase in the vials was frozen, an aliquot of approximately 200 µL from the DCM phase was taken rapidly and carefully to prevent any melting of the water phase and then placed into a GC vial. The GC method used to quantify 1,4-dioxane in DCM was the same as that described above, except that the injection volume was set at 3.0 µL. The lowest standard that was detectable was 80 µg/L (Figure 2.1b).

2.6.2 Fe(II)

Ferric iron reduction was monitored by quantifying the amount of dissolved Fe(II) with the ferrozine assay described by Stookey (1970). Samples (0.6 mL) of the liquid phase were withdrawn from microcosms after allowing the solids to settle out. The first 0.5 mL was discarded and the balance was discharged into a glass scintillation vial containing 4.9 mL of 0.5 N HCl. An aliquot (0.1 mL) of this acidified sample was withdrawn and discharged into 4.9 mL of the ferrozine reagent. Approximately 2 mL of the ferrozine solution was transferred to a cuvette (VWR). The absorbance was measured on a Cary 50 Bio Spectrophotometer at a wavelength of 562 nm. A typical standard curve is shown in Figure 2.2.

Due to low sample absorbance values with respect to standard absorbance values, sample size was increased to 1.5 mL. The first 0.5 mL of filtrate was discarded and the balance was discharged into a glass scintillation vial containing 4.0 mL of 0.61 N HCl. The subsequent steps were the same as described above.

Standard additions were used to evaluate matrix effects. Percent recovery was calculated following equation 2.1. Based on 18 samples evaluated, the average percent recovery was 98.1±11.2%, indicating no significant matrix interferences.

2.6.3 Sulfate and Nitrate

Sulfate was quantified by ion chromatographic analysis of dilute aqueous samples. Samples (1.0 mL) of the liquid phase were withdrawn from microcosms after allowing the solids to settle out. The water was passed through a syringe filter (0.2 μm PTFE, 13 mm diameter; VWR); the first 0.5 mL was discarded and the balance was

discharged into a 10 mL volumetric flask. The sample was then diluted with 9.5 mL of DDI water to obtain a 20 fold dilution. Diluted samples (0.5 mL) were discharged into vials (0.5 mL, PolyVial) vials and capped (0.5 mL, plain Polyvial). Filtered samples (25 μL) were injected onto a Dionex ICS-2100 ion chromatograph, equipped a 4-mm x 50 mm Dionex IonPac AG11 column (Thermo Scientific), and heated to 30°C. The suppression current was set to 48 mA. The eluent (4.5 mM Na_2CO_3; 0.8 mM $NaHCO_3$) was delivered at 1.0 mL/min. Sulfate eluted at 2.4 min. A typical standard curve is shown in Figure 2.3.

Standard additions were used to evaluate matrix effects. Percent recovery was calculated following equation 2.1. Based on 18 samples evaluated, the average percent recovery was 110.7±28.1, indicating no significant matrix interferences.

Nitrate was initially measured with the same ion chromatographic method as sulfate, although the background concentration of chloride interfered with nitrate quantification. Nitrate was subsequently determined using Hach method 8039, based on cadmium reduction. Samples (1.5 mL) were withdrawn from the liquid phase after allowing the solids to settle out. Samples were passed through a syringe filter (0.2 μm PTFE, 13 mm, VWR); the first 0.5 mL was discarded and the balance added to 9.0 mL of DDI water in a sample cell (25 mL, Hach). Cadmium reagent (NitraVer® 5 Nitrate Reagent Pillow, 10 mL) was added to the sample and allowed to react for 5 min before the absorbance measurement was taken on a Hach DR/890 colorimeter at a wavelength of 520 nm. A typical standard curve is shown in Figure 2.4.

2.6.4 VOCs and Oxygen

Volatile organic compounds (VOCs) were quantified by GC analysis of headspace samples (0.5 mL) and a Hewlett Packard 5890 Series II GC. Methane was quantified with a flame ionization detector and a 2.44-m x 3.175-mm column packed with 1% SP-1000 on 60/80 Carbopak B (Supelco). The carrier gas was nitrogen at 30 mL/min. The temperature program was 60 ºC isothermal for 2 min. Methane eluted at 0.4 min. The injector and detector temperatures were set at 200 ºC. The GC response to a headspace sample was calibrated to give the total mass of compound (*M*) in the serum bottle. Assuming the headspace and aqueous phases were in equilibrium, the total mass present was converted to an aqueous phase concentration:

$$C_l = \frac{M}{V_l + H_c V_g} \tag{2.2}$$

where C_l = concentration in the aqueous phase (µM); M = total mass present (µmol/bottle); V_l = volume of the liquid in the bottle (L); V_g = volume of the headspace in the bottle (L); and H_c = Henry's constant ((mol·m^{-3} gas concentration)/(mol·m^{-3} aqueous concentration)) at 23°C.

The method to quantify propane consumption was based on the same method as measuring methane, except the temperature program was 80 °C for 3 min.

1,1-DCE, vinyl chloride (VC), and ethene were also measured by the same method as methane, except the temperature program was 60 °C for 2 min, then increased to 150 °C at a rate of 20 °C/min, then increased to 170 °C at a rate of 10 °C/min for a total run time of 8.5 min. A typical standard curve for 1,1-DCE is shown in Figure 2.5.

Oxygen levels were monitored by injecting a 0.5 mL headspace sample onto a Hewlett Packard 5890 Series II GC equipped with a thermal conductivity detector and a 3.175-mm x 3.25-m 100/120 Carbosieve SII column (Supelco). Nitrogen was used as the carrier and reference gas at a rate of 50 mL/min. The temperature program was 105°C for 4 min, and the detector sensitivity was set to high. The injector and detector temperatures were set at 200°C.

2.6.5 ^{14}C Analysis

In order to evaluate the occurrence of 1,4-dioxane biodegradation, approximately 1 µCi of uniformly labeled material (specific activity of 50-60 mCi/mmol and a radiochemical purity of at least 97%; dissolved in acetone; Moravek Biochemicals) was added to the Set I and Set II Site I microcosms; none was added to the Site II microcosms since the supply of ^{14}C-1,4-dioxane was exhausted. Addition of 1 µCi per bottle added approximately 75 mg/L of acetone.

After adding the ^{14}C-1,4-dioxane, the initial amount present was determined by transferring samples (0.5 mL) of the liquid phase from each microcosm to liquid scintillation cocktail. To ascertain the amount of ^{14}C associated with 1,4-dioxane (versus possible impurities), liquid samples (1.0 mL) were withdrawn from the liquid phase after allowing solids to settle. The sample was passed through a filter (0.2 µm PTFE, 13 mm diameter; VWR); the first 0.65 mL was discarded and the balance was discharged into a vial (1.8 mL Kimble ROBO Vial™) with a 400 µL borosilicate glass insert (VWR) and then capped (PTFE/red silicone septum, Agilent Technologies). The filtered sample was injected (100 µL) onto an HPLC (Dionex UltiMate 3000) equipped with an Aminex®

HPX-87H column (300 mm x 7.8 mm, Biorad). The effluent from the column was collected and samples were added to liquid scintillation cocktail. Based on the retention time for 1,4-dioxane on this column, the ^{14}C collected in this interval was compared to the total ^{14}C injected onto the column. The average recovery of ^{14}C associated with 1,4-dioxane was 95±4.3%.

As the GC results for 1,4-dioxane will show, there was no compelling evidence for biodegradation in the anaerobic soil + groundwater microcosms. Consequently, ^{14}C transformation products were not evaluated.

CHAPTER THREE

RESULTS

Microcosms were prepared using soil and groundwater from industrial Sites I and II. Site I is in California and the contaminants include 1,4-dioxane and 1,1-DCE. Soil cores were collected 8.5-9.1 m, which was below the water table. Site II is in South Carolina and 1,4-dioxane the only contaminant of concern. The depth to the groundwater is less than 5 m.

3.1 Results for Site I

3.1.1 Set I Anaerobic Microcosms with Soil + Groundwater

Results for 1,4-dioxane in the Set I anaerobic microcosms are shown in Figure 3.1. The initial samples taken on day 8 were consistent with an initial target concentration of approximately 10 mg/L. Since the soil for Set I was likely exposed to oxygen during sample collection, these microcosms were evaluated only at the start and on days 234 and 416. Following more than 400 days of incubation, there was no evidence of a trend in 1,4-dioxane biodegradation in any of the treatments.

3.1.2 Set II Anaerobic Microcosms with Soil + Groundwater

Results for 1,4-dioxane in the Set II anaerobic microcosms are shown in Figure 3.2. The initial samples taken on day 9 were consistent with an initial target concentration of approximately 10 mg/L. Following 442-509 days of incubation, there was no evidence in support of 1,4-dioxane biodegradation in any of the treatments. Measurement of Fe(II) formation indicated that Fe(III) reduction occurred to a limited extent in the two treatments with Fe(III)-EDTA added (Figure 3.3). In the treatment

amended with sulfate, there was no indication of sulfate consumption (Figure 3.4), indicating a lack of a suitable electron donor and/or the absence of sulfate reducing bacteria. Methane formation was minor (Figure 3.5). The highest amount of methane formed (0.17 µmol/bottle) was less than 1% of the chemical oxygen demand (COD) of the 1,4-dioxane initially present.

On day 439, electron donor was added to the Fe(III) and sulfate amended microcosms (Figure 3.2). A stoichiometric amount of lactate and acetate was added to the sulfate and Fe(III) treatments, respectively, for complete reduction of the electron acceptors present. The 1,4-dioxane concentration in the Fe(III)-AQDS showed an initial decrease in relation to the other treatments, although no further decrease occured at the next sampling event on day 509.

3.1.3 Anaerobic Microcosms with Soil + MSM

Results for 1,4-dioxane in the anaerobic microcosms prepared with soil and MSM are shown in Figure 3.6. Following 329 days of incubation, there was no evidence in support of 1,4-dioxane biodegradation in the unamended or Fe(III)-EDTA treatments. Fe(III) reduction was observed in response to addition of Fe(III)-EDTA (Figure 3.7). Addition of acetate (40.3 mg/L, equivalent to a COD of 43.5 mg/L) on day 176 increased Fe(III) reduction, but with no apparent impact on 1,4-dioxane biodegradation. All of the nitrate added (254 mg/L) was consumed through day 188 (Figure 3.8), demonstrating the occurrence of denitrifying conditions; nevertheless, this did not stimulate anoxic biodegradation of 1,4-dioxane. Only a minor amount of sulfate consumption occurred through day 151 (Figure 3.9). Following the addition of lactate (231 mg/L, equivalent to

a COD of 249 mg/L) on day 176, nearly complete reduction of the sulfate added was observed. This indicated the soil contained sulfate reducing bacteria but was lacking in a biodegradable electron donor. As in the soil and groundwater microcosms, methane output in the soil + MSM microcosms was minor (Figure 3.10), amounting to approximately 2% of the COD of the 1,4-dioxane initially present.

Continued additions of lactate and electron acceptors to the sulfate and nitrate amended microcosms sustained reducing conditions in these treatments. A statistically significant decrease in 1,4-dioxane concentrations occurred in these microcosms since the onset of electron donor additions (Figure 3.11). The conversion ratio for dioxane disappearance versus sulfate reduced is 5.9×10^{-3} mg COD dioxane / mg COD SO_4^{2-}.

3.1.4 Aerobic Microcosms with Soil + Groundwater

Results for 1,4-dioxane in the aerobic microcosms prepared with soil and groundwater are shown in Figure 3.12. 1,4-Dioxane decreased by 33-39% in the unamended (i.e., no propane added, no bioaugmentation culture added) and propane amended treatments following 219 days of incubation. In contrast, the treatment with ENV487 added along with propane consumed three additions of 10-12 mg/L of 1,4-dioxane over a 55 day period. Propane and oxygen consumption was also highest in this treatment. These results suggest a limited amount of natural attenuation of 1,4-dioxane under aerobic conditions and a lack of a significant population of indigenous propanotrophs, based on the lack of significant propane consumption. The Site I microcosms did respond rapidly to addition of the ENV487 propanotrophic enrichment culture.

3.1.5 Effects of 1,1-DCE

By the time these microcosms were prepared, there was no longer a detectable level of VOCs present. To evaluate the potential impact of 1,1-DCE on propane biodegradation, approximately 1.0 mg/L was added (using DDI water saturated with 1,1-DCE) to the bioaugmented microcosms on day 62. This concentration was chosen based on the inhibitory effect of 1,1-DCE on 1,4-dioxane biodegradation by CB1190 (Mahendra, 2013). As shown in Figure 3.13, 1,1-DCE completely inhibited propane consumption. Presumably inhibition of propane consumption would have also inhibited cometabolism of 1,4-dioxane, although when 1,1-DCE was added there was no detectable level of 1,4-dioxane remaining. One day 114, the bottles were sparged in order to lower the 1,1-DCE concentration to 0.15 mg/L. Nevertheless, propane consumption remained inhibited. When 1,4-dioxane was added on day 134, the lack of propane consumption resulted in no significant consumption of 1,4-dioxane. 1,1-DCE was sparged from these bottles, and propane was added back to attempt to stimulate co-metabolic degradation. Propane and 1,4-dioxane consumption resumed, though at lower rates than experienced prior to the addition of 1,1-DCE. The presence of 1,1-DCE does appear to strongly inhibit propane consumption and consequently inhibits co-metabolism of 1,4-dioxane. This was further investigated in aerobic incubations of ENV487 with varying concentrations of 1,4-dioxane and 1,1-DCE.

The results from these incubations indicated that 1,4-dioxane and propane consumption were completely inhibited at 1,1-DCE concentrations above 0.1 mg/L for all concentrations of 1,4-dioxane (Figures 3.14 and 3.15). Consumption of 1,4-dioxane and

propane occurred in incubations with an initial 1,1-DCE concentration of 0.01 mg/L, suggesting that ENV487 is not inhibited at these levels. As shown in Figure 3.16, final concentrations of 1,1-DCE in these incubations ranged from 1.7 $x10^{-4}$ to $4.4x10^{-4}$ mg/L. Negligible degradation of 1,1-DCE occurred at concentrations above 0.1 mg/L. The presence of 1,4-dioxane had no discernible effects on the inhibition of propane consumption. The incubations containing 10 mg/L of 1,4-dioxane exhibited complete consumption of both propane and 1,4-dioxane. Based upon these results, a strategy to utilize anaerobic reductive dechlorinating bacteria to reduce 1,1-DCE concentrations to levels that do not inhibit the aerobic co-metabolism of 1,4-dioxane by ENV487 was developed.

Reductive dechlorination of 1,1-DCE was established after the inoculation of MicroCED, as suggested by the formation of VC and ethene (Figure 3.17). 1,1-DCE was the first compound to drop below detectable concentrations, followed by VC. Ethene accumulated in the microcosms, though was removed to below detection limits after the bottles were sparged with sterile air to re-establish aerobic conditions. 1,4-Dioxane concentrations were unaffected by the reductive dechlorination of 1,1-DCE. Following the re-establishment of aerobic conditions and inoculation of ENV487, rapid aerobic co-metabolism of 1,4-dioxane and metabolic metabolism of propane occurred. 1,4-Dioxane concentrations were reduced to below detectable limits within 15 days of inoculation in all microcosms (Figure 3.17).

3.2 Site II Results

3.2.1 Anaerobic Microcosms with Soil + Groundwater

Results for 1,4-dioxane in the Set I anaerobic microcosms are shown in Figure 3.18. Following 366 days of incubation, there was no evidence in support of 1,4-dioxane biodegradation in any of the treatments. Measurement of Fe(II) formation indicated that Fe(III) reduction occurred to a limited extent in the two treatments with Fe(III)-EDTA added (Figure 3.19). In the treatment amended with sulfate, the measured sulfate levels were below the level expected based on the concentration added; nevertheless, there was no trend in terms of sulfate reduction over time (Figure 3.20), indicating a lack of a suitable electron donor and/or the absence of sulfate reducing bacteria. Methane formation was minor in all but the unamended treatment, in which the accumulated amount (5.6 µmol/bottle) represented 39% of the COD of the 1,4-dioxane initially present (Figure 3.21). This indicated the presence of fermentable substrate other than 1,4-dioxane.

Additions of lactate and acetate were made to the sulfate and Fe(III) amended microcosms on day 340 (Figure 3.18). 1,4-Dioxane concentrations in all of these bottles remained unchanged at the next sampling event on day 366.

3.2.2 Anaerobic Microcosms with Soil + MSM

Results for 1,4-dioxane in the anaerobic microcosms prepared with soil and MSM are shown in Figure 3.22. Following 212 days of incubation, there was no evidence in support of 1,4-dioxane biodegradation in any of the treatments. Fe(III) was observed in response to addition of Fe(III)-EDTA (Figure 3.23). Addition of acetate (40.3 mg/L,

equivalent to a COD of 43.5 mg/L) on day 176 increased Fe(III) reduction, but with no apparent impact on 1,4-dioxane biodegradation (Figure 3.22). There was considerable variability in the nitrate measurements with respect to the amount of nitrate added (Figure 3.24), such that it is unclear if nitrate reduction was established. In the treatment amended with sulfate, the measured sulfate levels were below the level expected based on the concentration added; nevertheless, there was no apparent decreasing trend through day 151 (Figure 3.25). Following the addition of lactate (31.6 mg/L, equivalent to a COD of 34.2 mg/L) on day 176, there was a modest decrease in sulfate, suggesting that the soil contained sulfate reducing bacteria but was lacking in a biodegradable electron donor. As in the soil and groundwater microcosms, methane formation was appreciable in the unamended treatment; the accumulated amount (6.2 µmol/bottle) represented 44% of the COD of the 1,4-dioxane initially present (Figure 3.26). Methane formation was also significant in the sulfate-amended treatment, consistent with a lack of sulfate reduction. Methane formation indicated the presence of fermentable substrate other than 1,4-dioxane, which did not decrease while methane was increasing.

Continued additions of lactate and electron acceptors to the sulfate and nitrate amended microcosms sustained reducing conditions in these treatments. A statistically significant decrease in 1,4-dioxane concentrations occurred in these microcosms since the onset of electron donor additions (Figure 3.27). The conversion ratio for dioxane disappearance versus sulfate reduced is 9.9×10^{-3} mg 1,4-dioxane per mg sulfate, which is equivalent to 2.7×10^{-2} mg COD 1,4-dioxane / mg COD SO_4^{2-}.

3.2.3 Aerobic Microcosms with Soil + Groundwater

Results for 1,4-dioxane in the aerobic microcosms prepared with soil and groundwater are shown in Figure 3.28. 1,4-Dioxane was consumed below detection (i.e., 25 µg/L based on the micro-extraction method of sample preparation) in each of the treatments. The biodegradation rate was highest in the treatment with propane and culture added, followed by propane only and unamended. Presumably consumption of 1,4-dioxane in the propane amended treatments was due mainly to cometabolic activity. Consumption of 1,4-dioxane in the treatment without propane added indicates the presence of indigenous microbes capable of consuming 1,4-dioxane as a sole carbon and energy source. Alternatively, the soil may contain primary substrates whose aerobic biodegradation induces oxygenases capable of initiating fission of the 1,4-dioxane ring structure. The rate of propane consumption was highest in the bioaugmented treatment. Nevertheless, propane consumption in the treatment that was not bioaugmented confirmed the presence of indigenous propanotrophs. The rate of oxygen consumption followed the same trends as 1,4-dioxane.

Figure 3.29a summarizes the average rates of 1,4-dioxane biodegradation in the Site I and Site II aerobic microcosms. The rates were calculated by dividing the concentration of 1,4-dioxane consumed by the time required for the concentration to decrease below detection. Average rates in the Site I bioaugmented treatments were comparable to those in the Site II microcosms. The main difference was the lack of 1,4-dioxane biodegradation in the propane only and unamended treatments for Site I, whereas 1,4-dioxane was consumed in all of the aerobic treatments for Site II. Figure 3.29b

presents the average transformation yields (T_y), which were calculated by dividing the total amount of 1,4-dioxane consumed by the total amount of propane consumed. The higher transformation yield, for the Site II microcosms (0.40-0.68 mg 1,4-dioxane per mg propane) indicated a more efficient use of propane in terms of supporting cometabolism of 1,4-dioxane, even by the indigenous propanotrophs. This suggests the Site I microcosms contained contaminants in addition to 1,4-dioxane that inhibit the propanotrophs.

3.2.4 Results for Aerobic Enrichment of 1,4-Dioxane Metabolizing Bacteria

Results for 1,4-dioxane consumption in the aerobic enrichments prepared from the unamended Site II microcosms are shown in Figure 3.30. 1,4-Dioxane was consumed to below detectable limits by day 32 in all enrichments. A concentration of 20 mg/L was re-established and consumption continued at increased rates. The highest 1,4-dioxane concentration consumed in these enrichments was 80 mg/L. Four successful transfers of the enrichment culture have been made. The physical appearance of the biomass is opaque white flakes.

CHAPTER FOUR

DISCUSSION

1,4-Dioxane is generally regarded as recalcitrant under anaerobic conditions due to its stable structure and ether linkages. A microcosm study performed by Steffan et al. (2007) investigated biotransformation of 1,4-dioxane under iron, sulfate, and nitrate reducing conditions, as well as methanogenic conditions; no change in dioxane concentrations was noted in over 400 days of incubation. The only reported success in anaerobic degradation of dioxane by Shen et al. (2008) utilized anaerobic digester sludge, which contains a multitude of microbes and readily available electron donors not typically representative of aquifer conditions. The addition of humic acids increased the extent of 1,4-dioxane biodegradation in all treatments, with Fe(III)-EDTA amended incubations exhibiting further degradation than Fe(III) oxide amendments. While the conditions of readily degradable substrate in conjunction with Fe(III)-EDTA were replicated in this microcosm study for Sites I and II, no transformation of 1,4-dioxane occurred. This may be due in part to the intrinsic humic acid concentrations present in anaerobic digester sludge, which may be as high as 1.5% w/w of solids (Azman et al., 2015). The importance of humic acids to anaerobic biodegradation of ether containing compounds is further delineated by the results for anaerobic biodegradation of methyl tert-butyl ether (MTBE) (Finneran and Lovley, 2001). Only treatments that received Fe(III)-EDTA and humic acid amendments degraded MTBE, with the humic acids speculated to act as shuttles that facilitate external electron transfer. The presence of

readily degradable substrate and humic acids coincide with the possible 1,4-dioxane disappearance that occurred in the Site I Set II Fe(III)-AQDS amended microcosms following the addition of lactate on day 439 (Figure 3.2); additional monitoring would be required to confirm a trend in 1,4-dioxane biodegradation.

The apparent anaerobic biodegradation of 1,4-dioxane in the sulfate and nitrate amended MSM microcosms (Figures 3.11 and 3.27) may be attributable to anaerobic co-metabolism. Similar to non-specific oxygenase enzymes degrading aliphatic compounds in aerobic environments, non-specific enzymes induced during the anaerobic metabolism of lactate could be responsible for the disappearance of 1,4-dioxane in these microcosms. The anaerobic co-metabolic biodegradation of benzothiophene by a sulfate reducing culture grown on naphthalene as a primary substrate demonstrates a process similar to the one presumed to be responsible for 1,4-dioxane degradation in this microcosm study (Annweiler et al., 2001). The enzyme induced to degrade naphthalene targeted benzothiophene to produce both 2- and 5-carboxybenzothiophene, indicative of non-specific conversion of the non-growth substrate (Annweiler et al., 2001). A pathway for anaerobic cometabolic degradation of 1,4-dioxane has not yet been established.

The aerobic co-metabolism experiments show that the ENV487 mixed culture is effective at degrading 1,4-dioxane below detectable limits. One metric for performance of the culture is the ratio of the mass of 1,4-dioxane consumed to the mass of primary substrate consumed. T_y values for a variety of substrates and non-growth substrates are summarized in Table 4.1. Values for propanotrophs other than this study were not found. The closest comparison is 1.4 mg 1,4-dioxane per mg of tetrahydrofuran.

A significant challenge for aerobic bioremediation of 1,4-dioxane is its frequent co-occurrence with 1,1-DCE, which has a strong inhitory effect on microbes that utilize oxygenase. This is a consequence of the reactivity of 1,1-DCE with oxygenases and the generation of 1,1-dichloroethene epoxide, which is a potent toxin. The effect of 1,1-DCE on the initial rates of 1,4-dioxane cometabolism by ENV487 is compared in Figure 4.1 (modified from Mahendra et al., 2013) to those of *P. dioxanivorans* CB1190, *P. mendocina* KR1, and a toluene monooxygenase expressing *E. coli* strain. The initial rates for ENV487 as a function of 1,1-DCE and 1,4-dioxane concentrations were determined from Figure 3.14. Similar to other cometabolic dioxane degraders, dioxane consumption rates were severely hindered even after removal of 1,1-DCE, suggesting that exposure to this compound causes irreversible damage to either cells or enzymes responsible for co-metabolism.

The inhibitory effects of 1,1-DCE may be mitigated by first anaerobically degrading the compound using reductive dechlorinating bacteria. Due to the negative correlation found between 1,4-dioxane attenuation and chlorinated solvent concentrations at many field sites (Adamson et al., 2015), this remediation strategy may provide an effective means to treat 1,4-dioxane plumes that co-occur with chlorinated solvents. The results from this study indicated that aerobic co-metabolic bacteria functioned effectively following reductive dechlorination of 1,1-DCE to ethene, suggesting that the anaerobic treatment of this compound may reduce it to concentrations that will no longer affect aerobic degradation of dioxane.

Evidence that aerobic bacteria capable of using 1,4-dioxane as a sole source of carbon and energy are widely distributed is mounting. Although such microbes were not found in Site I microcosms, they were for Site II. The fact that the enrichment was transferred four times with 1,4-dioxane as the sole source of carbon and energy provides strong evidence that the enrichment contains microbes that are growing on this compound. The identity of the microbe is not yet known. However, when the enrichment was compared visually to an actively growing culture of CB1190, there were distinct similarities.. The enrichment culture was dominated by opaque grey to white flakes that adhered to the glass surface at the air and medium interface. This is similar to the appearance of CB1190 following growth on 1,4-dioxane, suggesting that the microbe responsible for 1,4-dioxane in the enrichment culture may also be a *Pseudonocardia* spp. The occurrence of natural 1,4-dioxane attenuation typically corresponds to the presence of genes expressing monooxygenase enzymes similar to those found in CB1190 (Lippincott et al., 2015).

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Biodegradation of 1,4-Dioxane in Site I Microcosms

- Biodegradation of 1,4-dioxane did not occur in any of the anaerobic treatments prepared with Site I soil and groundwater. A limited amount of Fe(III) reduction was observed, but no discernable level of sulfate reduction or methanogenesis, suggesting the soil and groundwater lacked bioavailable electron donor.
- A notable decrease in the 1,4-dioxane concentration in the Fe(III)-AQDS amended microcosms following an addition of acetate suggests the potential of anaerobic biodegradation. Additional monitoring is needed to verify this observation.
- Biodegradation of 1,4-dioxane did not occur in any of the anaerobic treatments prepared with Site I soil and MSM prior to electron donor additions. Fe(III) reduction occurred to a limited extent; sulfate reduction occurred only in response to lactate addition, indicating the soil contains sulfate reducing bacteria. There was no significant level of methane production. These results further confirmed that the Site I soil lacks bioavailable electron donor. Furthermore, the results suggest that anaerobic treatment of 1,4-dioxane at Site I is not likely occurring at a significant rate.
- A statistically significant decrease in 1,4-dioxane concentrations occurred in the sulfate and nitrate amended microcosms following sustained lactate additions.

Enrichments have been prepared with slurries from the sulfate amended bottles to investigate this phenomenon further.

- 1,4-Dioxane decreased by 33-39% in the unamended (i.e., no propane added, no bioaugmentation culture added) and propane amended aerobic microcosms following 219 days of incubation. In contrast, the treatment with ENV487 added along with propane consumed three additions of 10-12 mg/L of 1,4-dioxane over a 55 day period. Propane and oxygen consumption was also highest in this treatment. These results suggest a limited amount of natural attenuation of 1,4-dioxane occurs under aerobic conditions; however, the soil and groundwater lack indigenous propanotrophs, based on the lack of significant propane consumption. The Site I microcosms did respond rapidly to the addition of the ENV487 propanotrophic enrichment culture.
- In the aerobic microcosms amended with ENV487 and propane, cometabolism of 1,4-dioxane was inhibited by 1,1-DCE at 0.15 and 1.0 mg/L. The propanotrophic culture exhibited no significant cometabolic activity on 1,1-DCE. When the 1,1-DCE was removed through sparging, 1,4-dioxane and propane consumption resumed, though at a lower rate than prior to the introduction of 1,1-DCE. These results suggest that aerobic cometabolism of 1,4-dioxane by propanotrophs will be inhibited unless 1,1-DCE is removed first.
- Anaerobic biodegradation of 1,1-DCE with reductive dechlorinating bacteria followed by aerobic cometabolism of 1,4-dioxane with propanotrophs indicated a possible remediation strategy for sites where chlorinated solvents inhibit cometabolic

activity. 1,4-Dioxane was consumed within 16 days of establishing aerobic conditions and inoculating with ENV487, suggesting that the concentration of 1,1-DCE was reduced to non-inhibitory levels under anaerobic conditions.

5.2 Biodegradation of 1,4-Dioxane in Site II Microcosms

- Biodegradation of 1,4-dioxane did not occur in any of the anaerobic treatments prepared with Site II soil and groundwater. A limited amount of Fe(III) reduction was observed, but no discernable level of sulfate reduction. Methanogenesis was observed in the unamended treatment, suggesting the presence of fermentable substrate other than 1,4-dioxane, which did not decrease while methane was increasing.
- Biodegradation of 1,4-dioxane did not occur in any of the anaerobic treatments prepared with Site II soil and MSM prior to electron donor additions. Fe(III) reduction and sulfate reduction were evident only in response to addition of acetate or lactate; nitrate reduction was variable. Methane formation was appreciable in the unamended treatment, indicating the presence of fermentable substrate other than 1,4-dioxane, which did not decrease while methane was increasing.
- A statistically significant decrease in 1,4-dioxane concentrations occurred through sustained electron donor and acceptor additions in the sulfate and nitrate amended microcosms. Enrichments have been prepared with slurries from the sulfate amended bottles to investigate this phenomenon further.
- Aerobic biodegradation was evident in all treatments, indicating the presence of indigenous microbes with the ability to biodegrade 1,4-dioxane under aerobic

conditions. Treatments amended with propane and ENV487 exhibited the most rapid degradation of 1,4-dioxane, followed by the propane amended and unamended treatments, respectively. In the propane and propane plus culture amended samples evaluated by the micro-extraction method, 1,4-dioxane concentrations were below the 80 μg/L detection limit. These results suggest that aerobic biodegradation of 1,4-dioxane may be a viable treatment approach for Site II.

- Aerobic enrichments of the unamended Site II microcosms successfully degraded multiple additions of 1,4-dioxane through multiple transfers. This indicates the presence of indigenous bacteria capable of utilizing 1,4-dioxane as a growth substrate under aerobic conditions. Work is underway to isolate and characterize these microbes.

5.3 Recommendations

Based on the findings of this research, the following recommendations are proposed:

- Further additions of acetate should be made to the Site I Set II Fe(III)-AQDS amended microcosms to confirm the occurrence of 1,4-dioxane biodegradation.
- Enrichments from the sulfate and nitrate amended MSM microcosms should be monitored to verify the occurrence of anaerobic cometabolic biodegradation of 1,4-dioxane.
- The threshold concentration at which 1,1-DCE is not inhibitory to ENV487 should be established.

- The aerobic 1,4-dioxane metabolizing bacteria associated with Site II should be isolated and characterized.

TABLES

Table 2.1. Sources and purity of chemicals.

Chemical	Source	Purity
1,4-dioxane	Aldrich	99.9%
^{14}C-1,4-dioxane	Moravek Biochemicals	-[a]
Propane	Airgas	99.5%
1,1-DCE	Acros	99.9%
DCM	OmniSolv	99.9%
VC	Matheson	99.9%
Ethene	AirGas	99.9%
Fe(III)-EDTA	J.T. Baker	67.5%
AQDS	Pfaltz & Bauer	-[b]
Sodium sulfate	EMD Chemicals	99%
Sodium nitrate	Sigma	99%
Resazurin	J.T. Baker	80%

[a] Radiochemical purity of at least 97%; specific activity of 50-60 mCi/mmol; dissolved in acetone.

[b] Not available.

Table 2.2. Initial concentrations of 1,4-dioxane in Site I samples.

	1,4-dioxane (mg/L)	
Sample Type	**Set I**	**Set II**
Groundwater, Well 1	0.91	1.2
Groundwater, Well 2	0.96	0.4
Groundwater, Well 3	-[a]	-
Groundwater, Well 4	-	-
Soil	-	-

[a]Sample concentrations were below detection limit.

Table 4.1. Summary of transformation yield (T_y) values.

Growth Substrate	**Contaminant Transformed**	**T_y (mg contaminant mg substrate^{-1})**	**Reference**
Tetrahydrofuran (THF)	1,4-dioxane	1.4[a]	Zenker et. al., 2002
Methane	Chloroform	0.01	Kim et. al, 1997
Propane	Chloroform	0.01-0.015	Kim et. al., 1997
Butane	Chloroform	0.01-0.026	Kim et. al., 1997
Butane	1,1,1-Trichloroethane	0.04	Jitnuyanont et. al., 2001
Phenol	Trichloroethylene	0.062-0.11	Hopkins et. al., 1993
Methane	Trichloroethylene	0.034-0.040	Smith and McCarty, 1997
Methane	Vinyl Chloride	1.0-3.5[a]	Anderson and McCarty, 1997
Methane	*trans*-Dichloroethene	3.8[b]	Anderson and McCarty, 1997

[a] Calculated based on the transformation capicity (2.5 mg 1,4-dioxane/mg TSS) and yield (0.56 mg TSS/mg THF).
[b] Calculated from values expressed as mol contaminant mol substrate^{-1}.

FIGURES

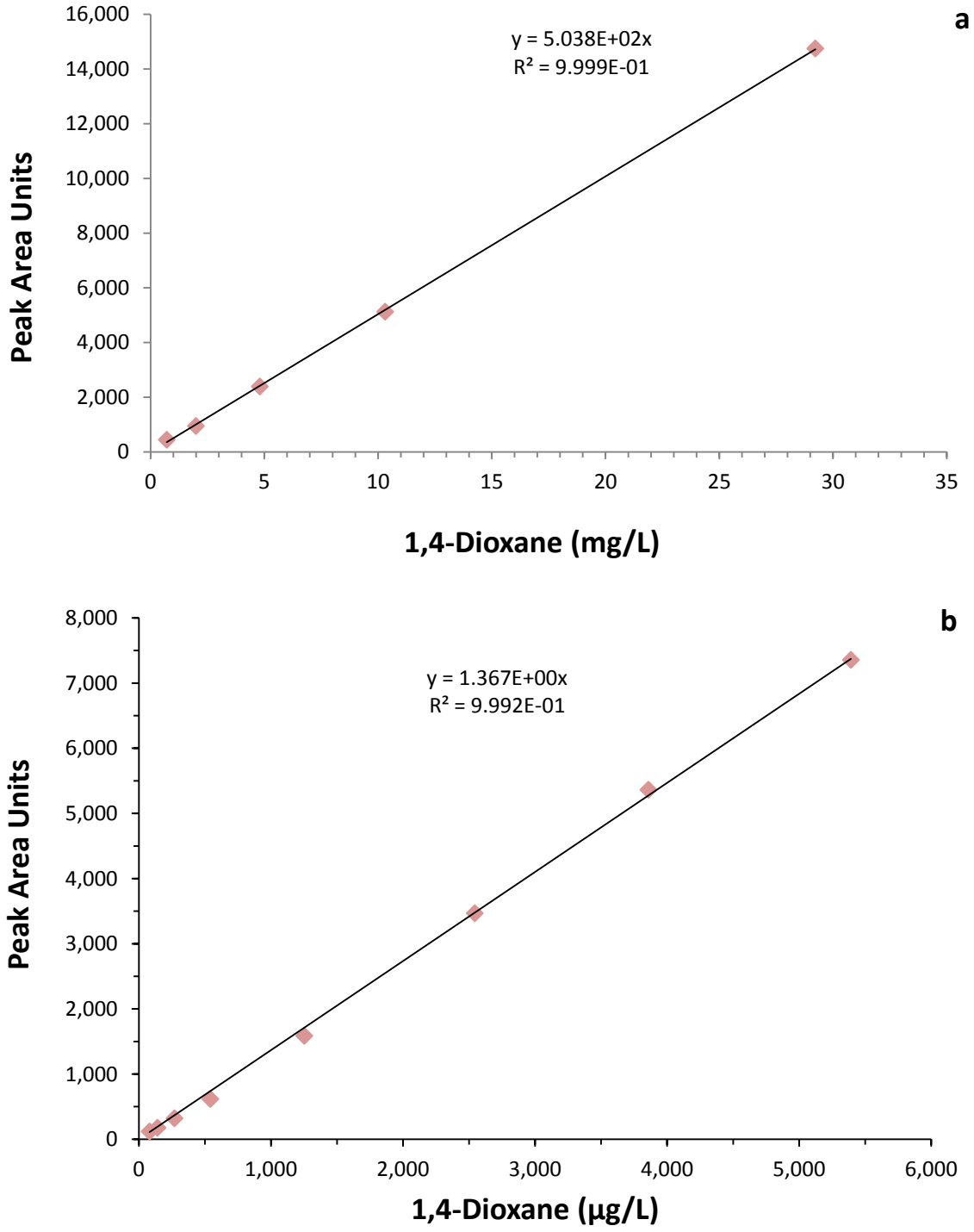

Figure 2.1. Representative standard curve used to determine response factor for GC analysis of 1,4-dioxane: **a)** direct aqueous injections; and **b**) micro-frozen extraction method.

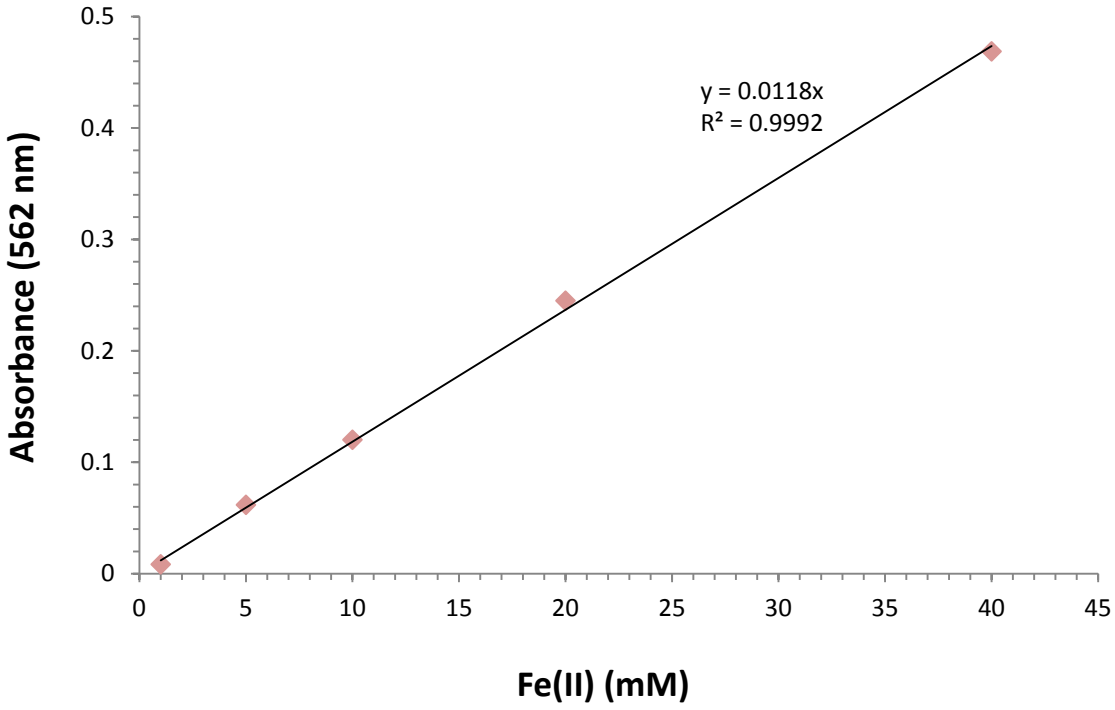

Figure 2.2. Representative standard curve used to determine response factor for Ferrozine assay analysis of Fe(II).

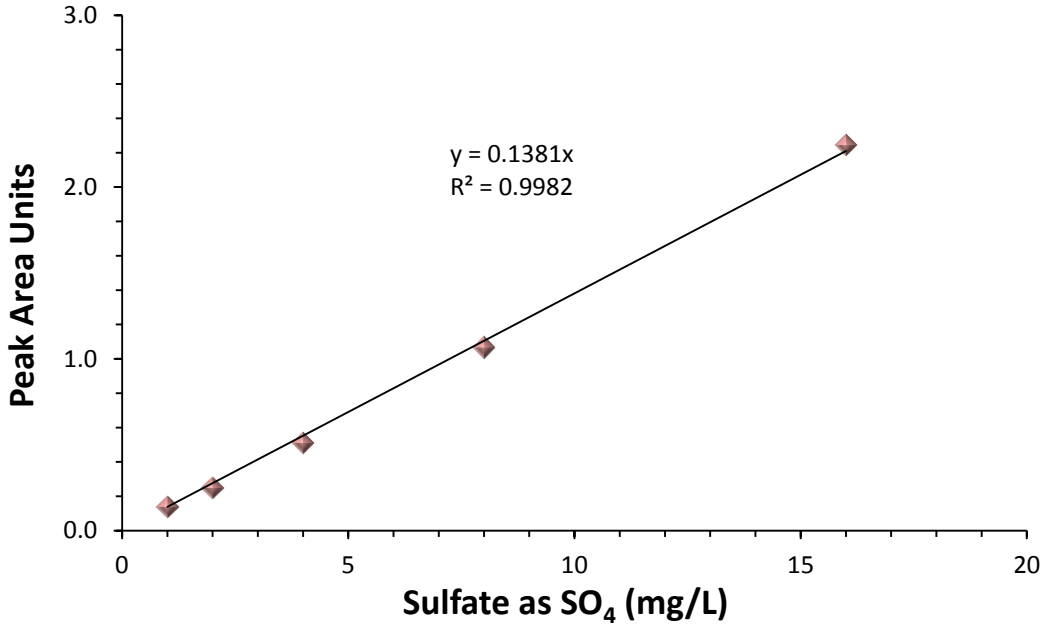

Figure 2.3. Representative standard curve used to determine response factor for IC analysis of sulfate.

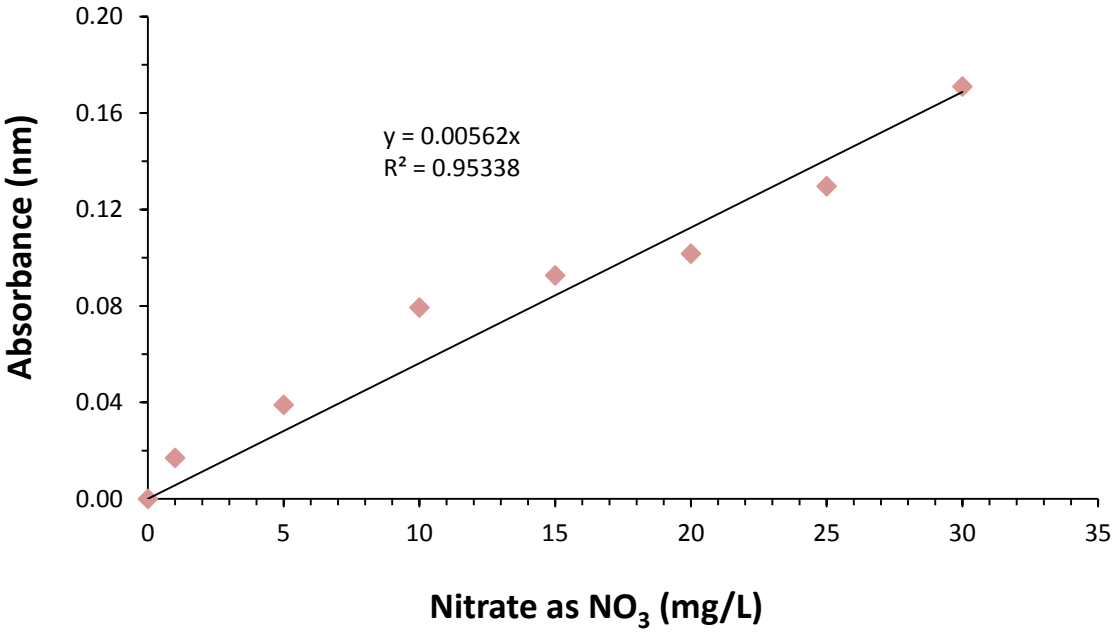

Figure 2.4. Representative standard curve used to determine response factor for spectrophometric analysis of nitrate.

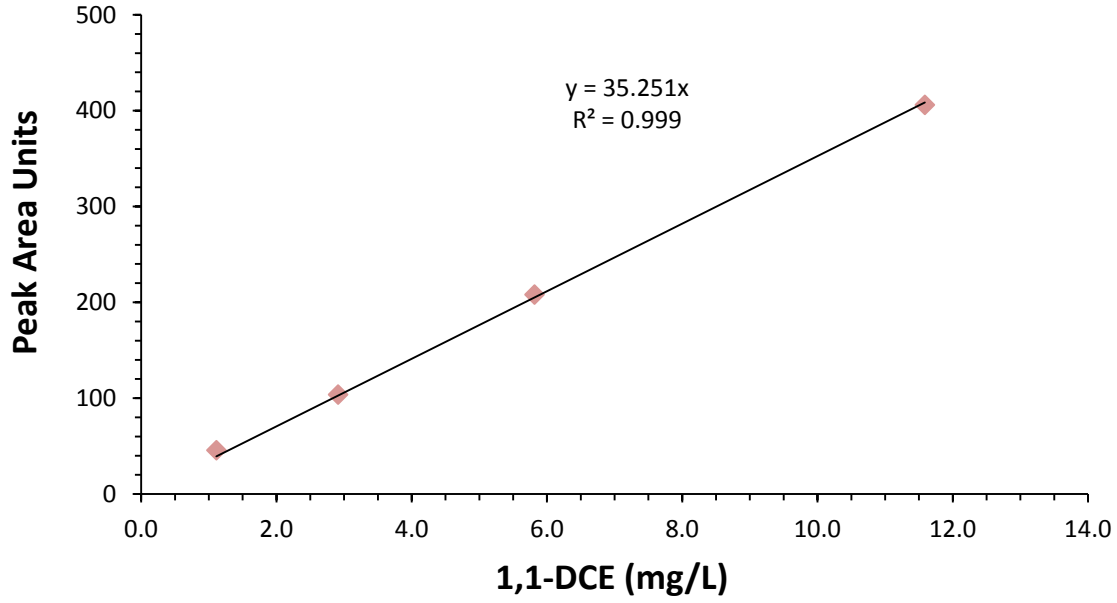

Figure 2.5. Representative standard curve used to determine response factor for GC analysis of VOCs (1,1-DCE shown).

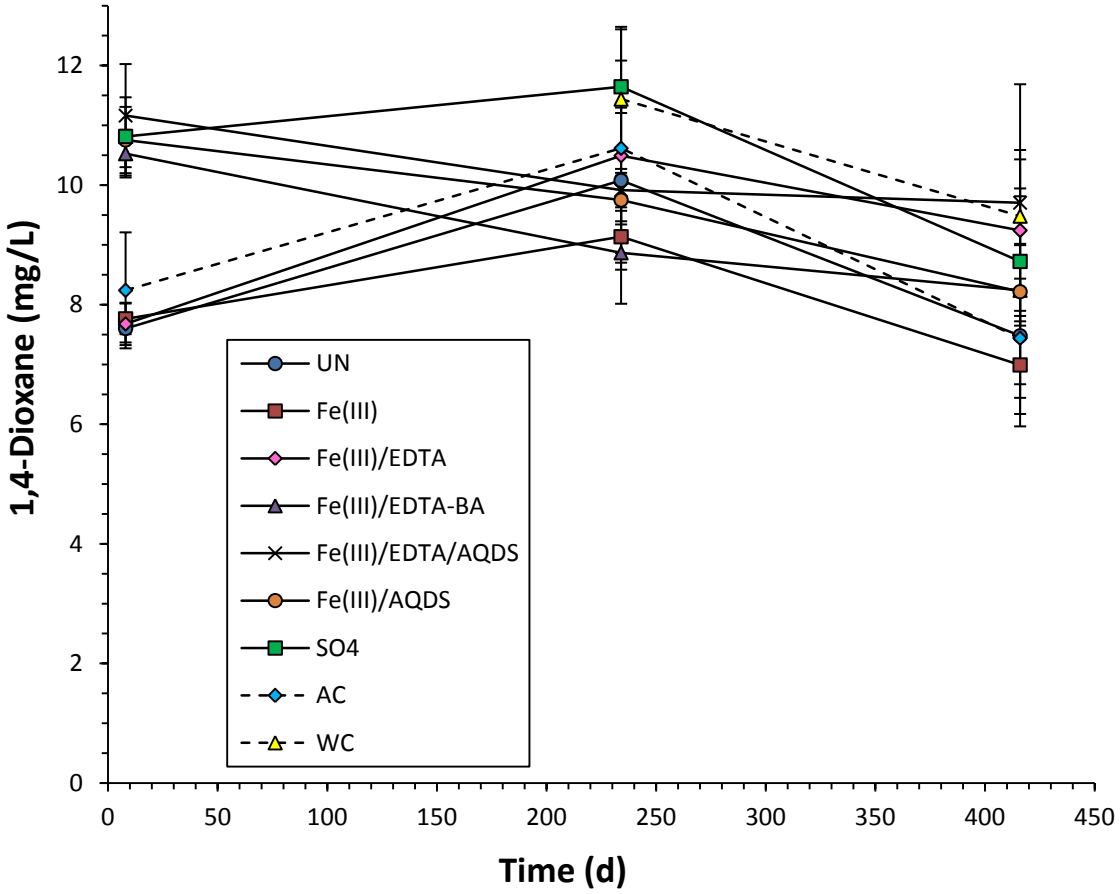

Figure 3.1. 1,4-Dioxane results for the Site I Set I anaerobic microcosms prepared with soil and groundwater. Data shown are the averages for triplicate bottles. Error bars represent standard deviations.

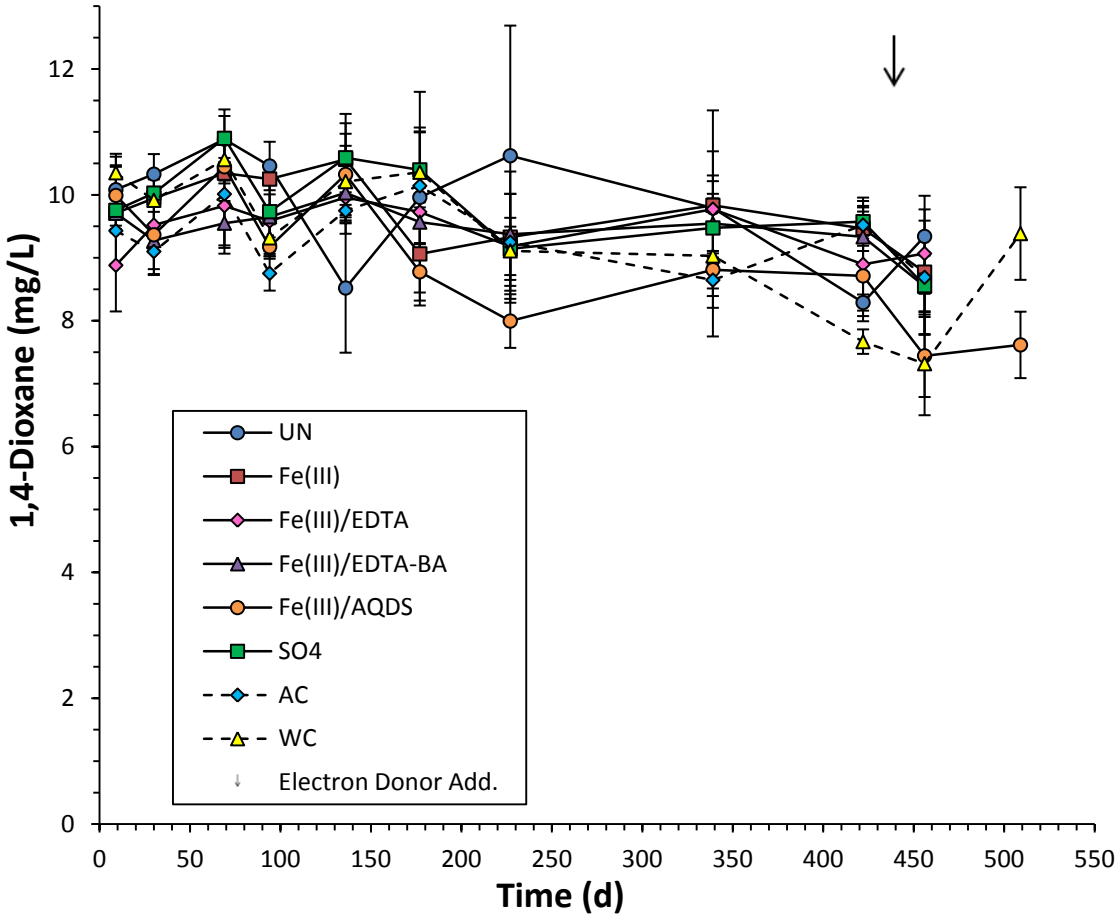

Figure 3.2. 1,4-Dioxane results for the Site I Set II anaerobic microcosms prepared with soil and groundwater. Data shown are the averages for triplicate bottles. Error bars represent standard deviations. The arrow represents the addition of electron donor to iron and sulfate amended bottles.

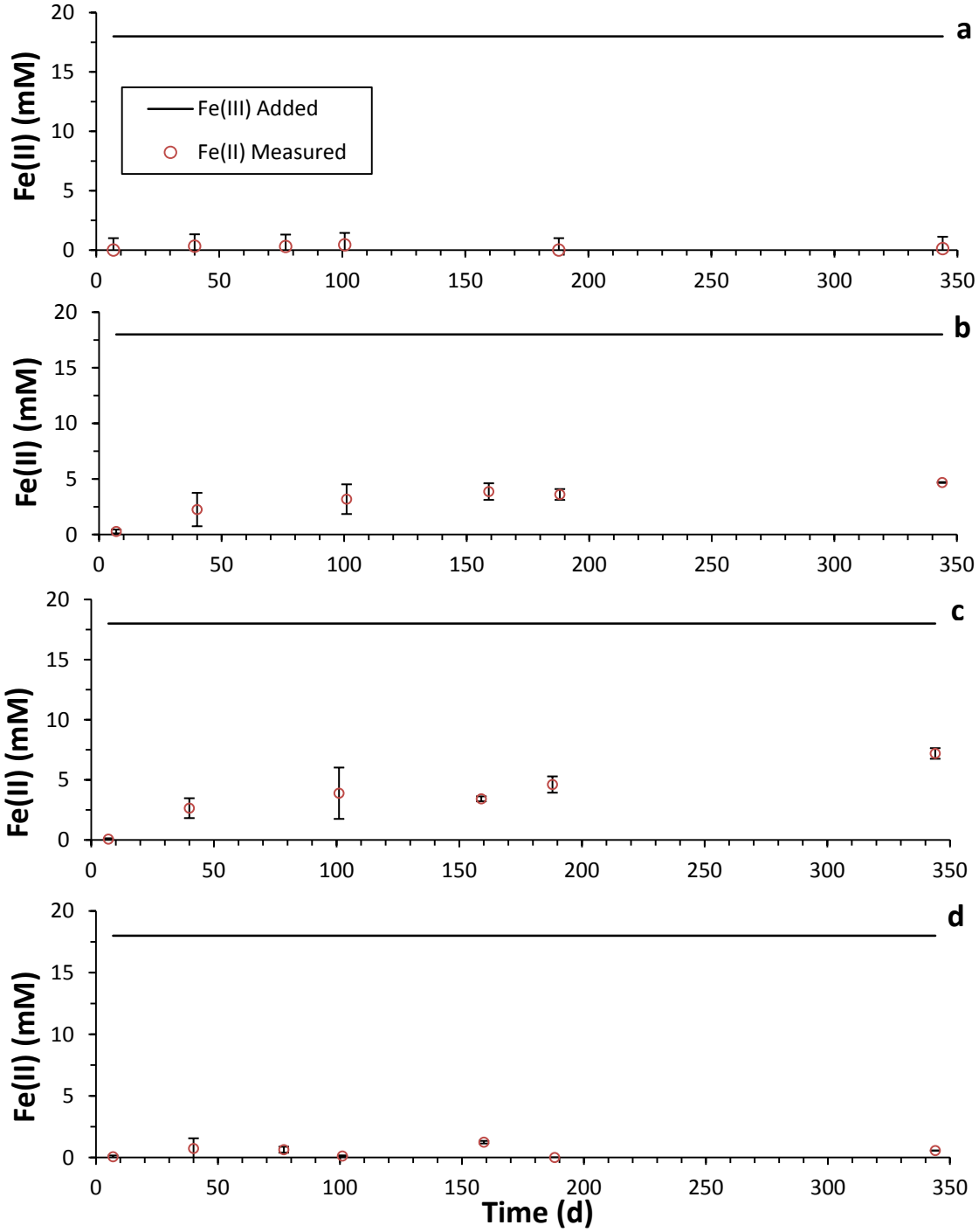

Figure 3.3. Ferrous iron results for Site I Set II anaerobic microcosms prepared with soil and groundwater with Fe(III) added as **a**) Fe(III)-gel; **b**) Fe(III)-EDTA; **c**) Fe(III)-EDTA + inoculum from microcosms exhibiting 1,4-dioxane transformation; and **d**) Fe(III)-gel + AQDS. Data points are the average measured Fe(II) level for triplicate bottles; error bars represent standard deviations. Solid lines represent the concentration of Fe(III) added.

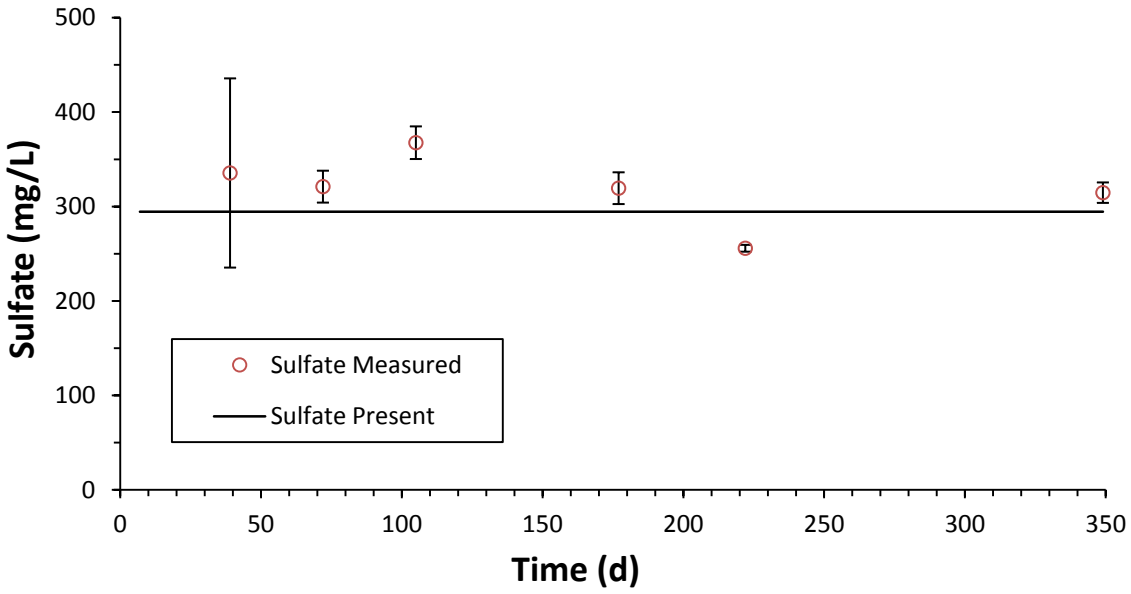

Figure 3.4. Sulfate results for Site I Set II anaerobic microcosms prepared with soil and groundwater. Data points represent measured sulfate concentrations; error bars are standard deviations. The solid line represents the calculated amount of sulfate initially present plus the amount added.

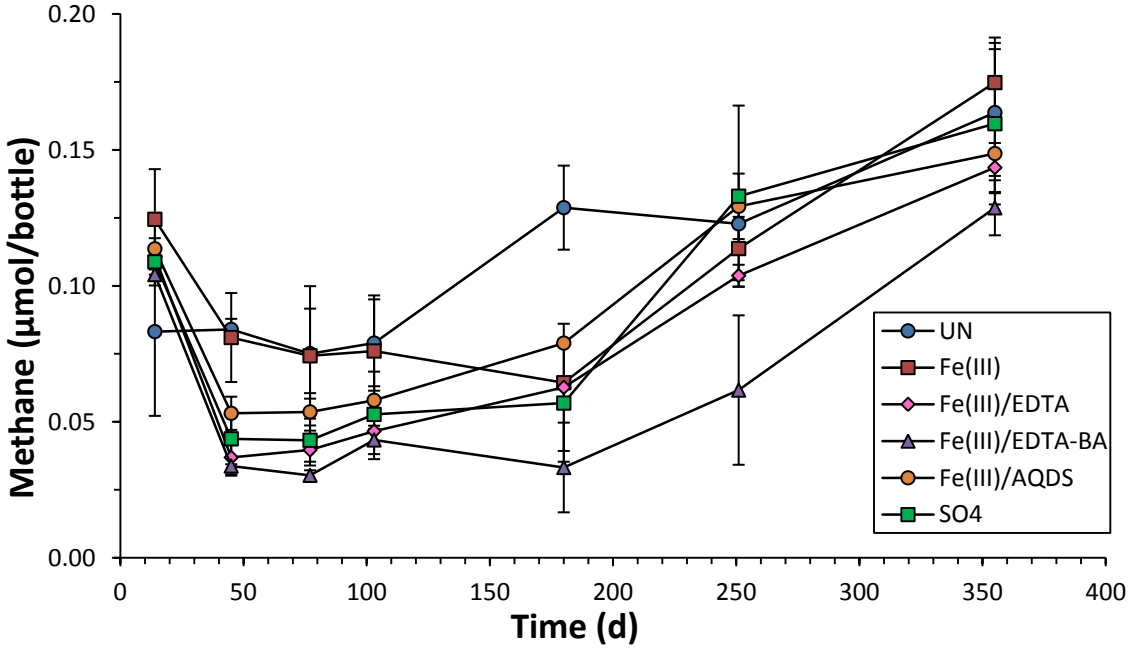

Figure 3.5. Methane production results for Site I Set II anaerobic microcosms prepared with soil and groundwater. Data shown are averages for triplicate bottles; error bars represent standard deviations.

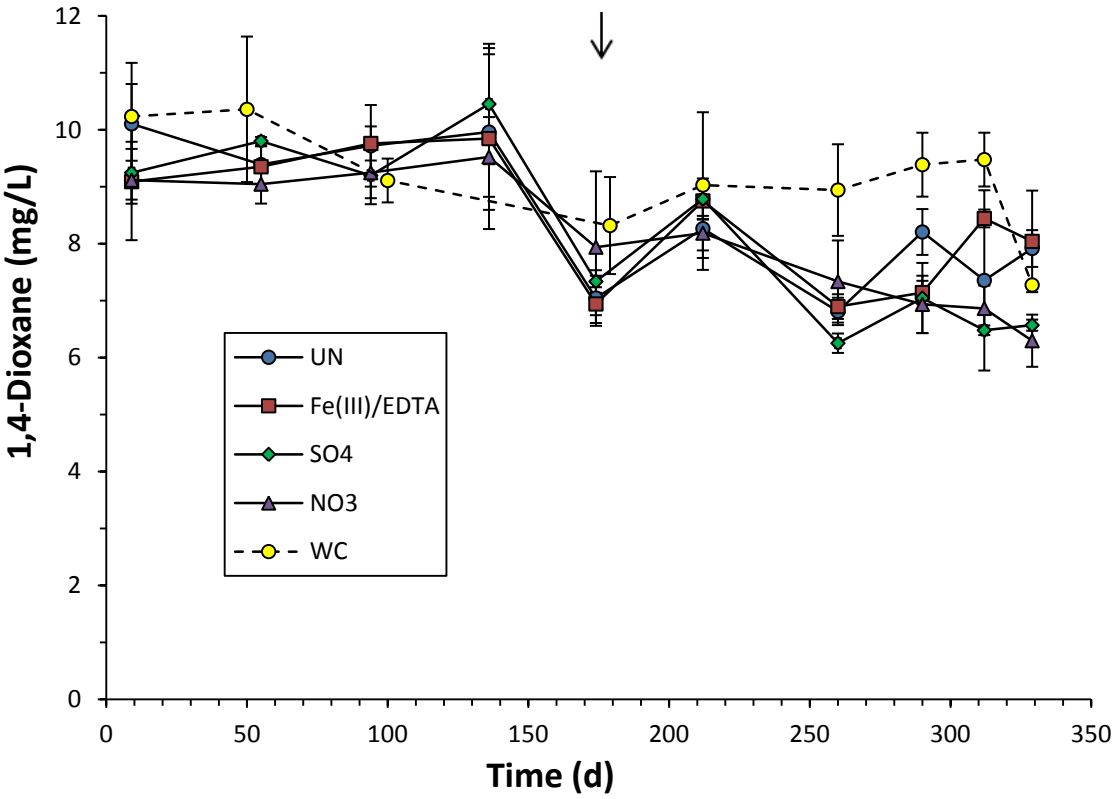

Figure 3.6. 1,4-Dioxane results for the Site I anaerobic microcosms prepared with soil and MSM. Data shown are the averages for triplicate bottles; error bars represent standard deviations. The arrow represents the addition of electron donor to iron and sulfate amended bottles.

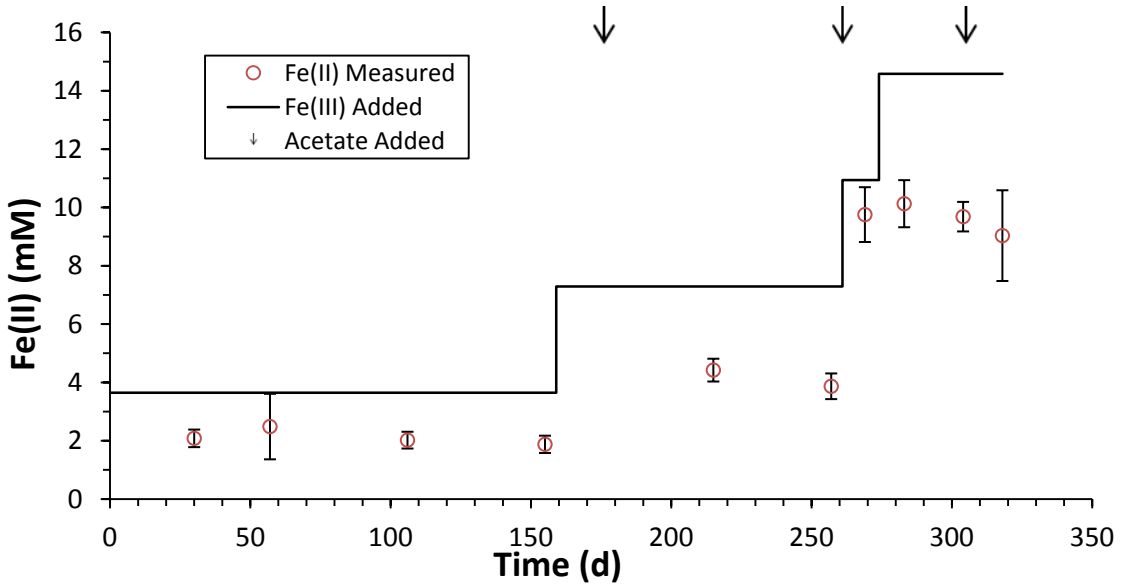

Figure 3.7. Ferrous iron results for Site I anaerobic microcosms prepared with soil and MSM. Data points are the average measured Fe(II) level for triplicate bottles; error bars represent standard deviations. The solid line represents the cumulative amount of Fe(III)-EDTA added; the arrow represents addition of acetate.

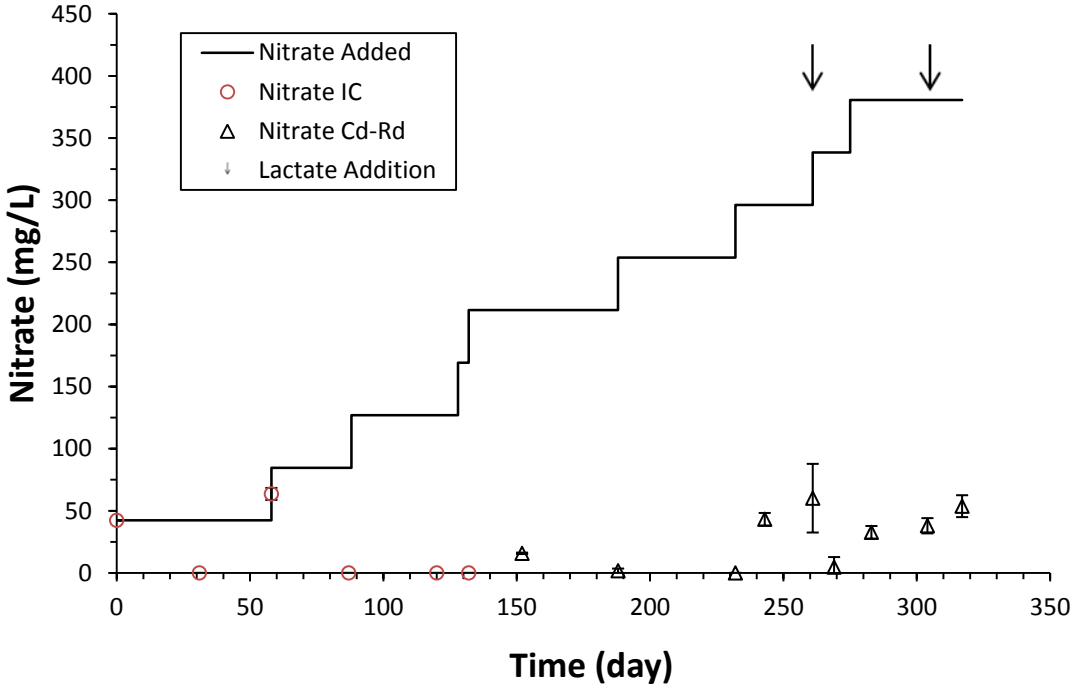

Figure 3.8. Nitrate results for Site I anaerobic microcosms prepared with soil and MSM. Data points are the average measured nitrate level for triplicate bottles; error bars represent standard deviations. The solid line represents the cumulative concentration of nitrate added.

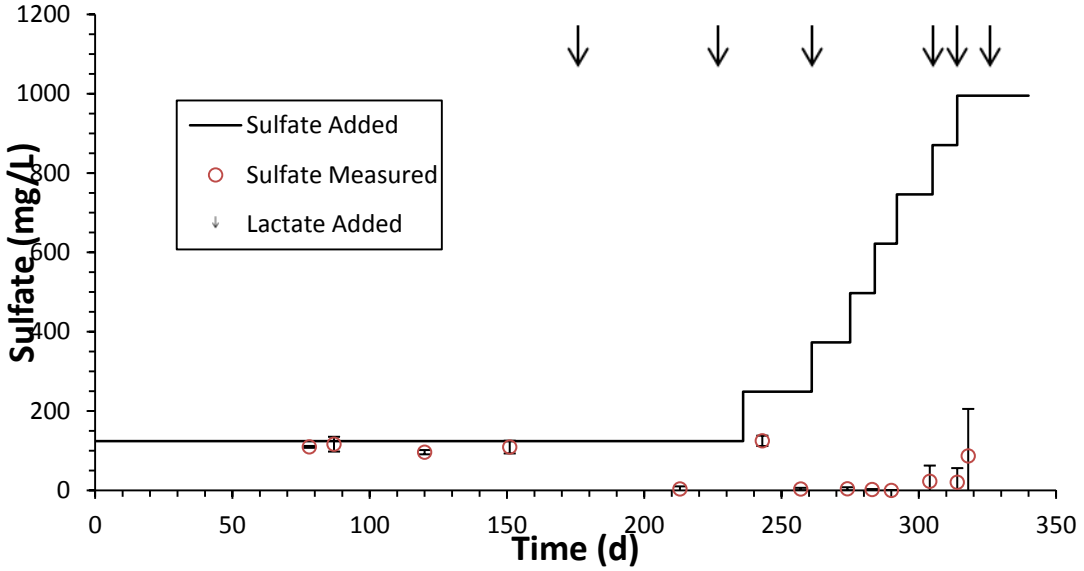

Figure 3.9. Sulfate results for Site I anaerobic microcosms prepared with soil and MSM. Data points represent average level in triplicate bottles; error bars are standard deviations. The solid line represents the calculated amount of sulfate initially present plus the amount added; the arrow denotes addition of lactate.

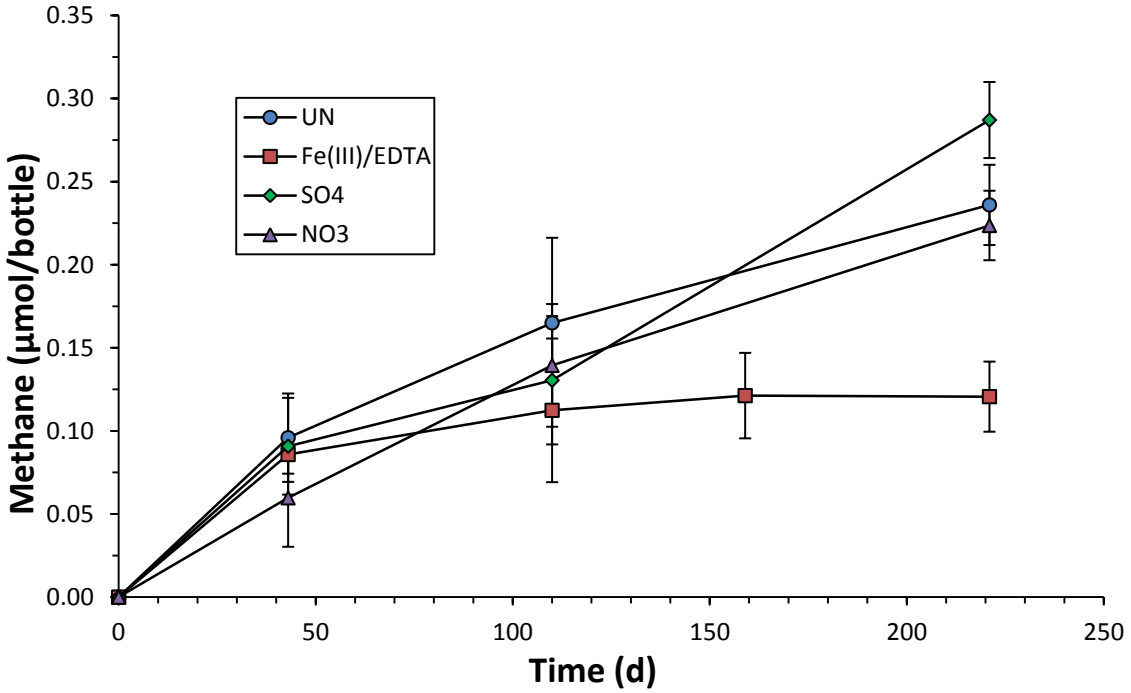

Figure 3.10. Methane production results for Site I anaerobic microcosms prepared with soil and MSM. Data shown are averages for triplicate bottles; error bars represent standard deviations.

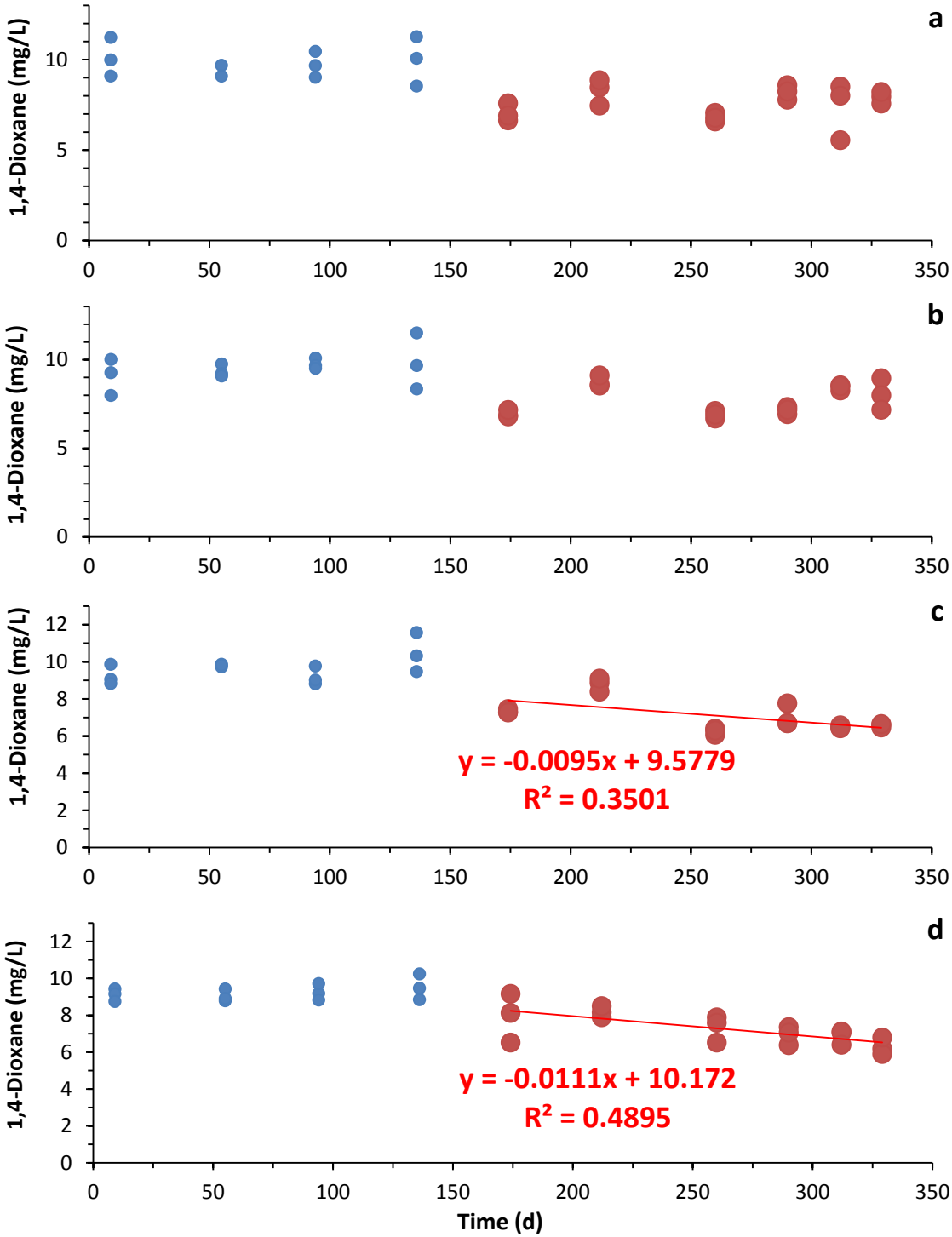

Figure 3.11. Linear regression analysis of Site I MSM microcosms. Statistically significant negative slopes were obtained for the analysis of **c)** sulfate and **d)** nitrate. No significant decrease was obtained for the **a)** unamended and **b)** Fe(III)-EDTA amended microcosms.

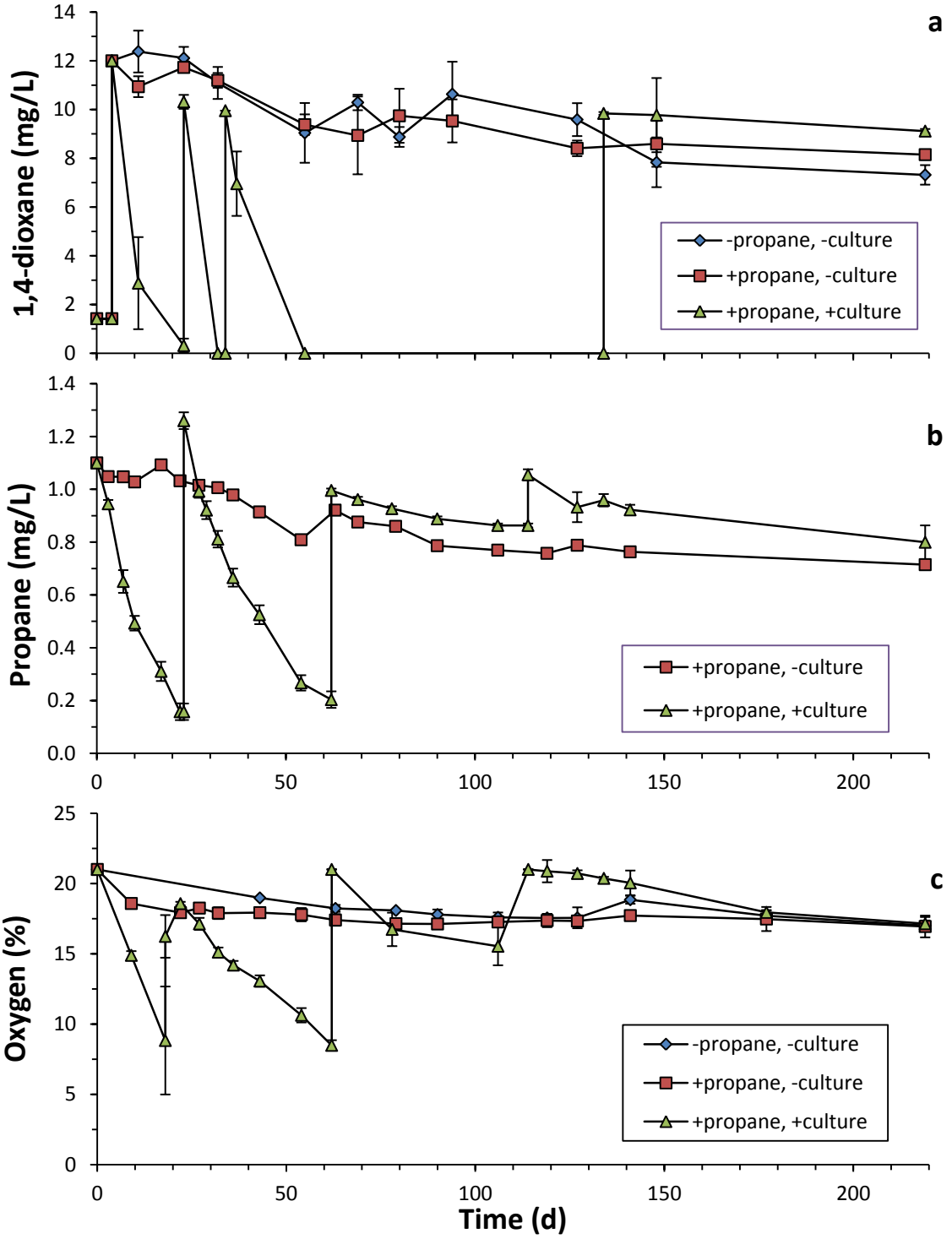

Figure 3.12. Results for Site I aerobic microcosms prepared with soil and groundwater; **a)** 1,4-dioxane; **b)** propane; and **c)** percent oxygen present in microcosm headspace. Data shown are averages for triplicate bottles; error bars represent standard deviations.

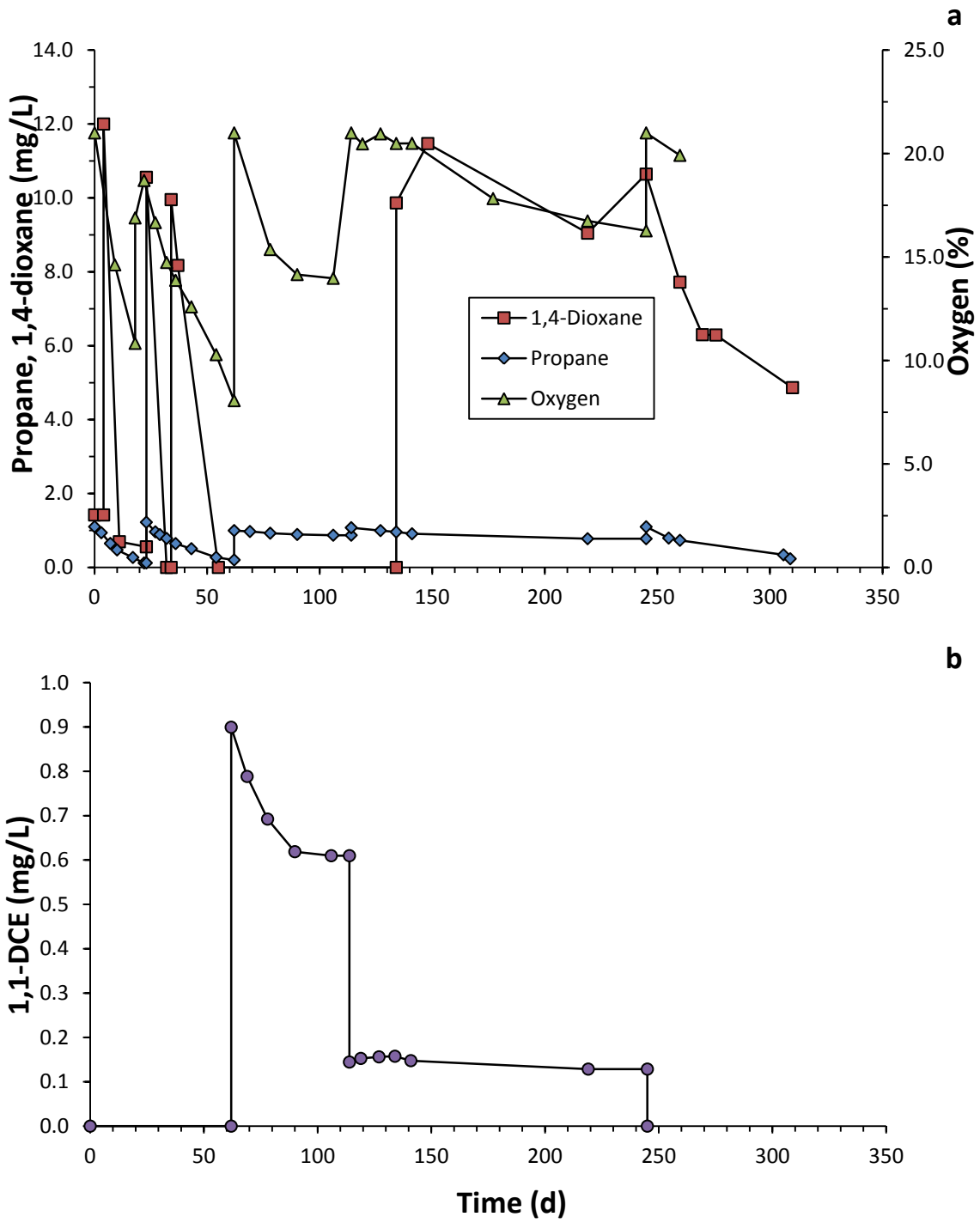

Figure 3.13. Results for an individual Site I aerobic microcosm prepared with soil and groundwater, amended with propane + inoculated with propanotrophs; 1,1-DCE was added on day 62; **a)** 1,4-dioxane, propane, and oxygen; **b)** 1,1-DCE. On day114 the groundwater was sparged with room air and the microcosm was respiked with a lower concentration of 1,1-DCE. On day 245 1,1-DCE was removed.

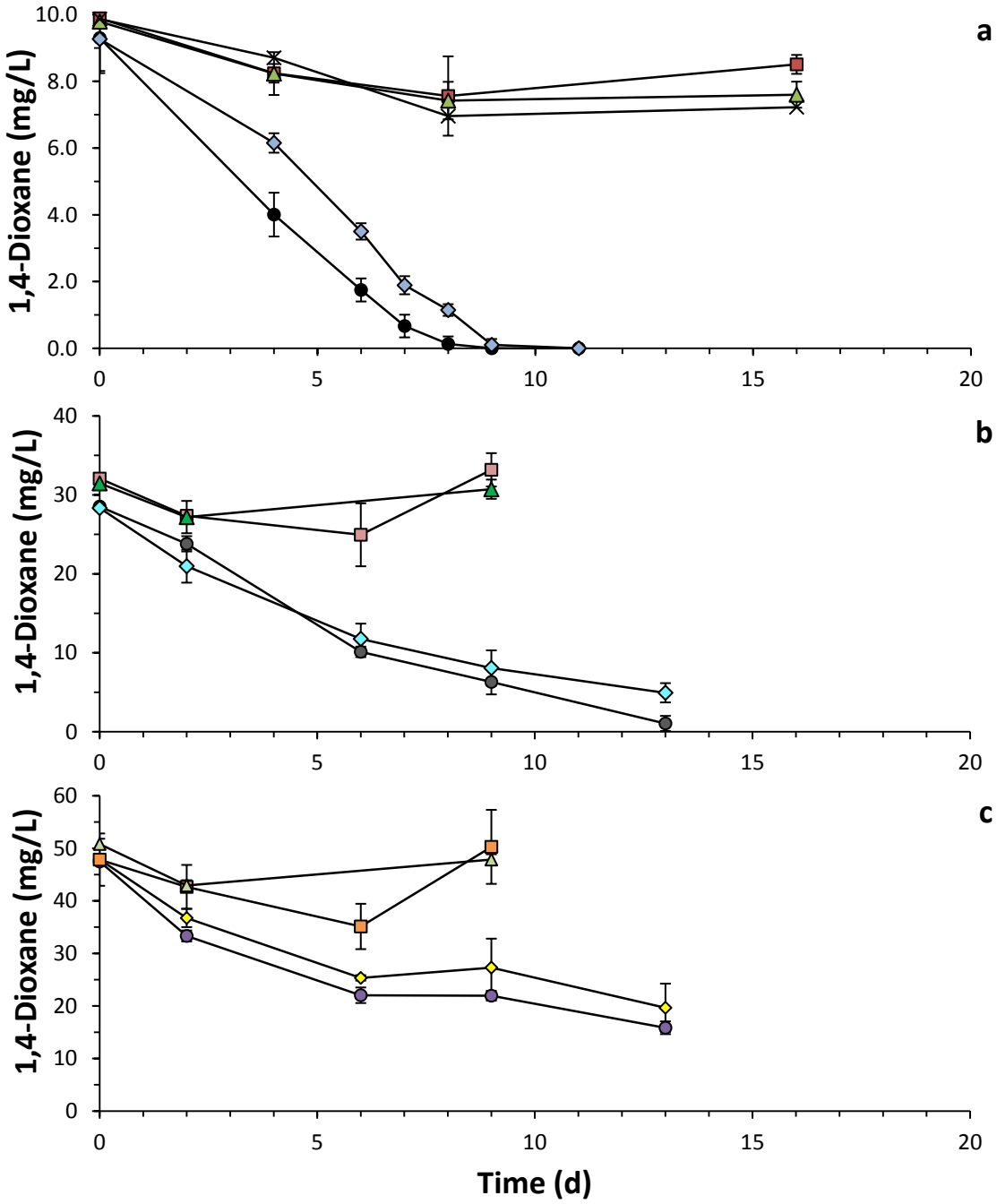

Figure 3.14. Results for 1,1-DCE inhibition incubations prepared with glass beads and BSM with an initial 1,4-dioxane concentration of **a)** 10 mg/L, **b)** 30 mg/L, and **c)** 50 mg/L. Concentrations of 1,1-DCE in each series: circles - 0 μM; diamonds - 0.1 μM; squares - 1.0 μM; triangles - 10 μM; crosses - 10 μM sterile control. Data shown are averages for triplicate bottles; error bars represent standard deviations.

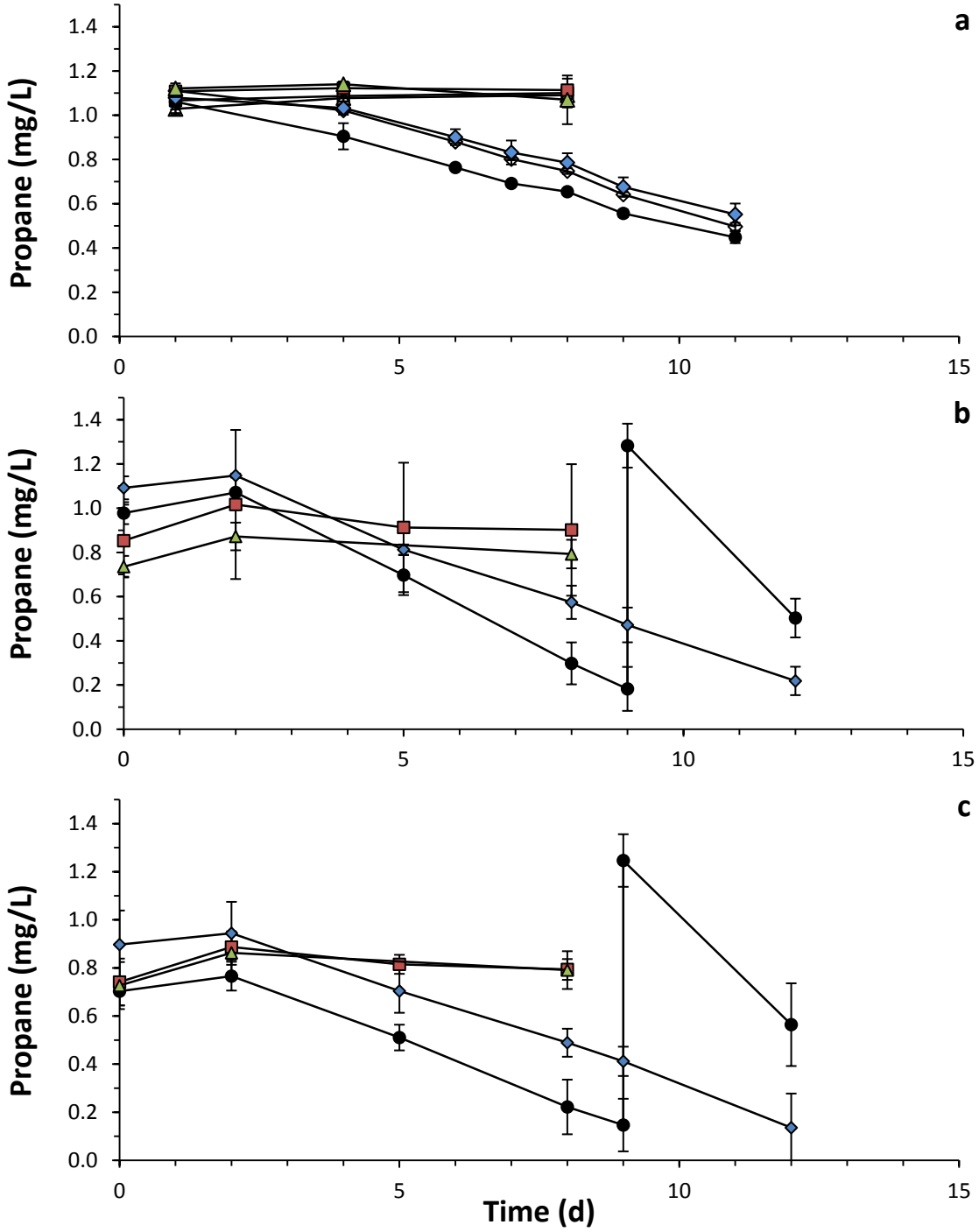

Figure 3.15. Propane results for 1,1-DCE inhibition incubations with an initial 1,4-dioxane concentration of **a)** 10 mg/L, **b)** 30 mg/L, and **c)** 50 mg/L. Concentrations of 1,1-DCE in each series: circles - 0 μM; diamonds - 0.1 μM; squares - 1.0 μM; triangles - 10 μM. Open series in **a)** represent incubations with no 1,4-dioxane. Data shown are averages for triplicate bottles; error bars represent standard deviations.

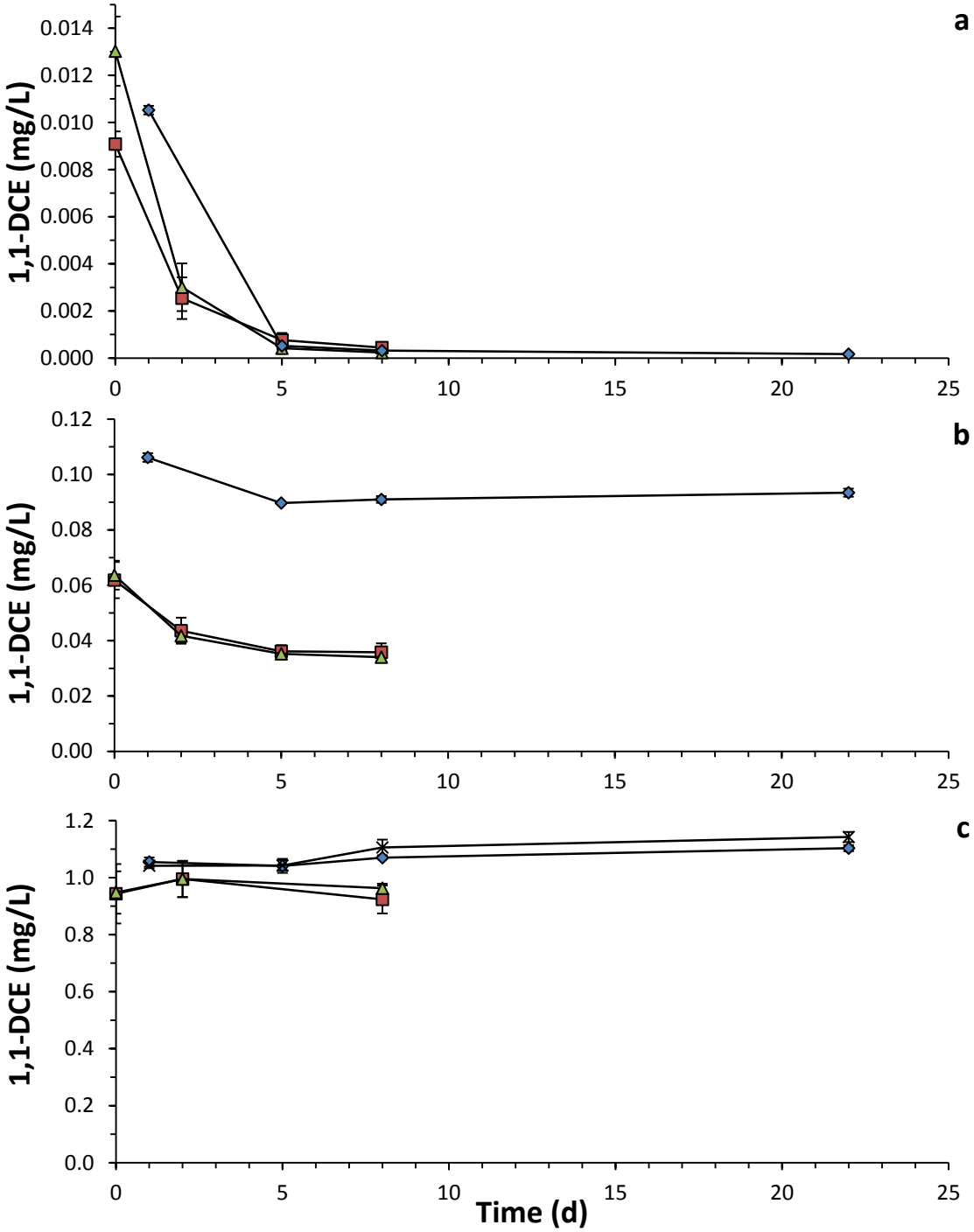

Figure 3.16. 1,1-DCE results for 1,1-DCE inhibition incubations for **a)** 0.01 mg/L, **b)** 0.1 mg/L, and **c)** 1.0 mg/L aqueous concentrations. Concentrations of 1,4-dioxane in each series: diamonds - 10 mg/L; squares - 30 mg/L; triangles - 50 mg/L; crosses - sterile control. Data shown are averages for triplicate bottles; error bars represent standard deviations.

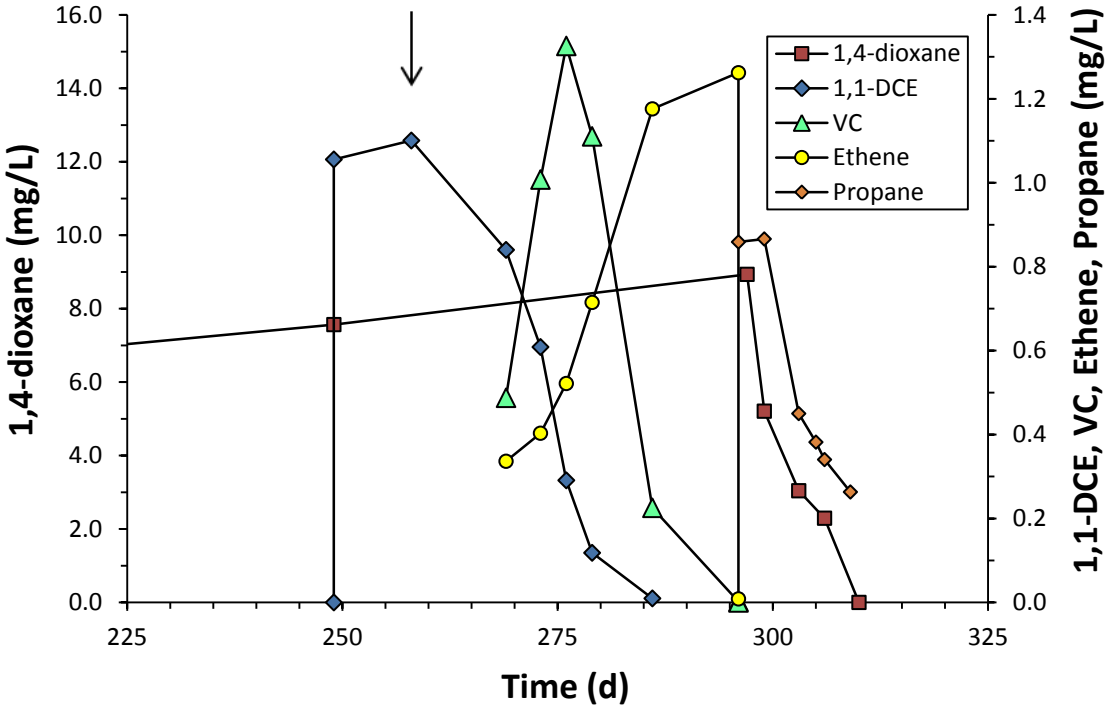

Figure 3.17. Results for the anaerobic degradation of 1,1-DCE and subsequent co-metablic degradation of 1,4-dioxane. On day 249, 1,1-DCE was added to the microcosms. The black arrow denotes the MicroCED inoculation on day 258. After VC was reduced below detection limits, aerobic conditions were established and propane and ENV487 were added.

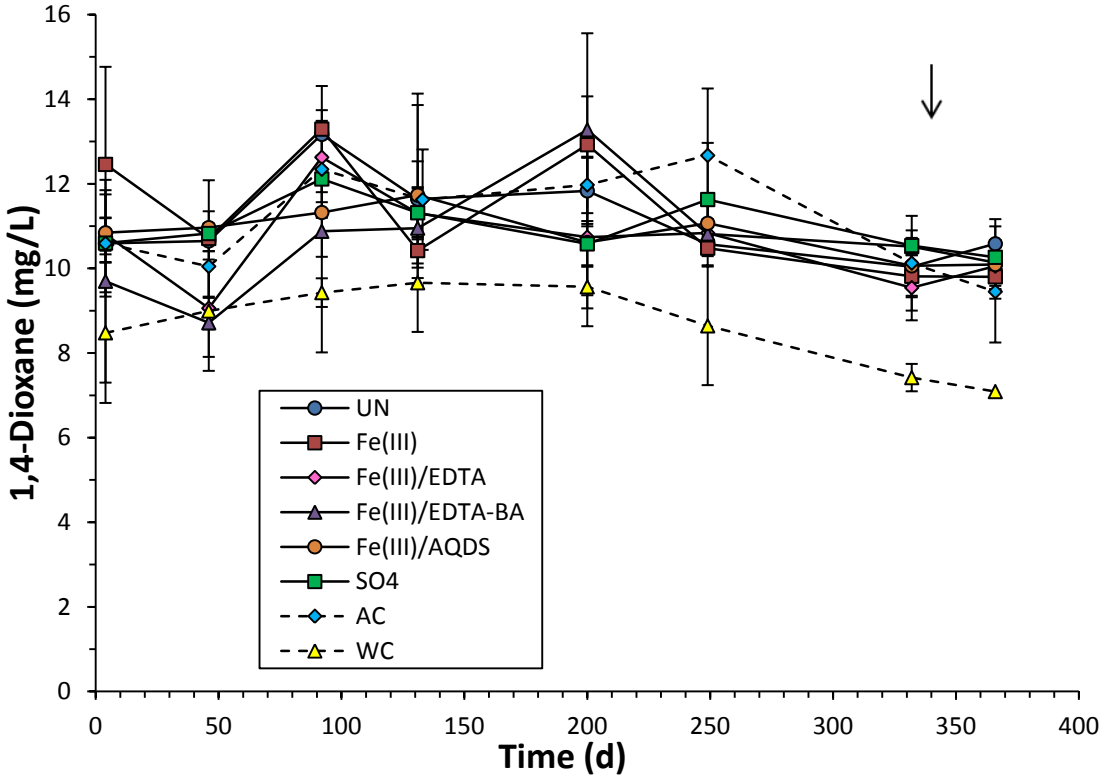

Figure 3.18. 1,4-Dioxane results for the Site II anaerobic microcosms prepared with soil and groundwater. The arrow indicates the start of electron donor additions. Data shown are the averages for triplicate bottles; error bars represent standard deviations.

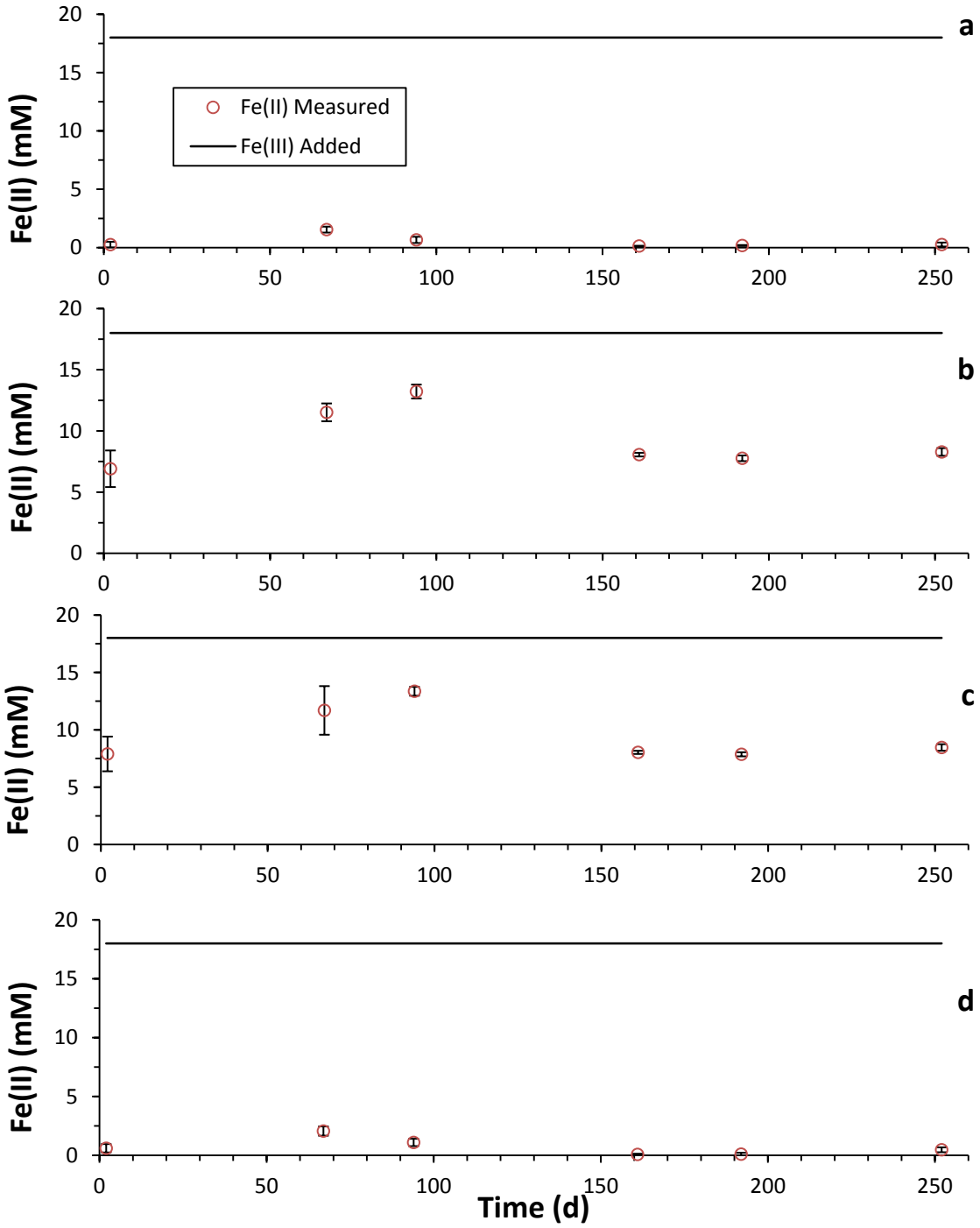

Figure 3.19. Ferrous iron results for Site II anaerobic microcosms prepared with soil and groundwater with Fe(III) added as **a**) Fe(III)-gel; **b**) Fe(III)-EDTA; **c**) Fe(III)-EDTA + inoculum from microcosms exhibiting 1,4-dioxane transformation; and **d**) Fe(III)-gel + AQDS. Error bars represent standard deviations for triplicate bottles. Solids lines represent the Fe(III) added.

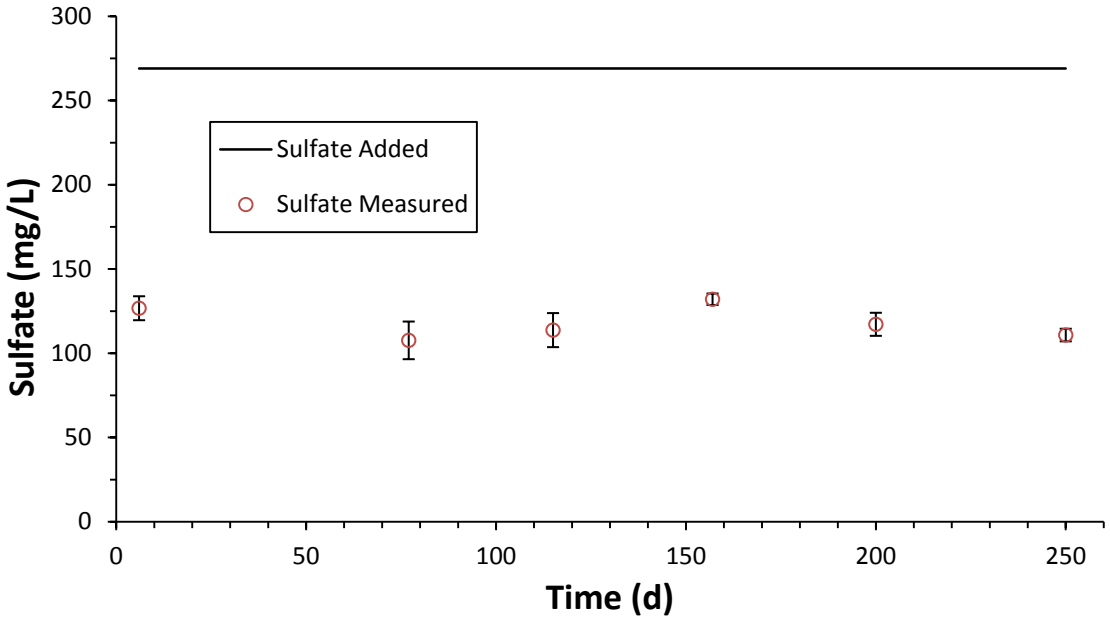

Figure 3.20. Sulfate results for Site II anaerobic microcosms prepared with soil and groundwater. Data points represent average sulfate concentrations for triplicate bottles; error bars represent the standard deviation. The solid line represents the calculated amount of sulfate initially present plus the amount added.

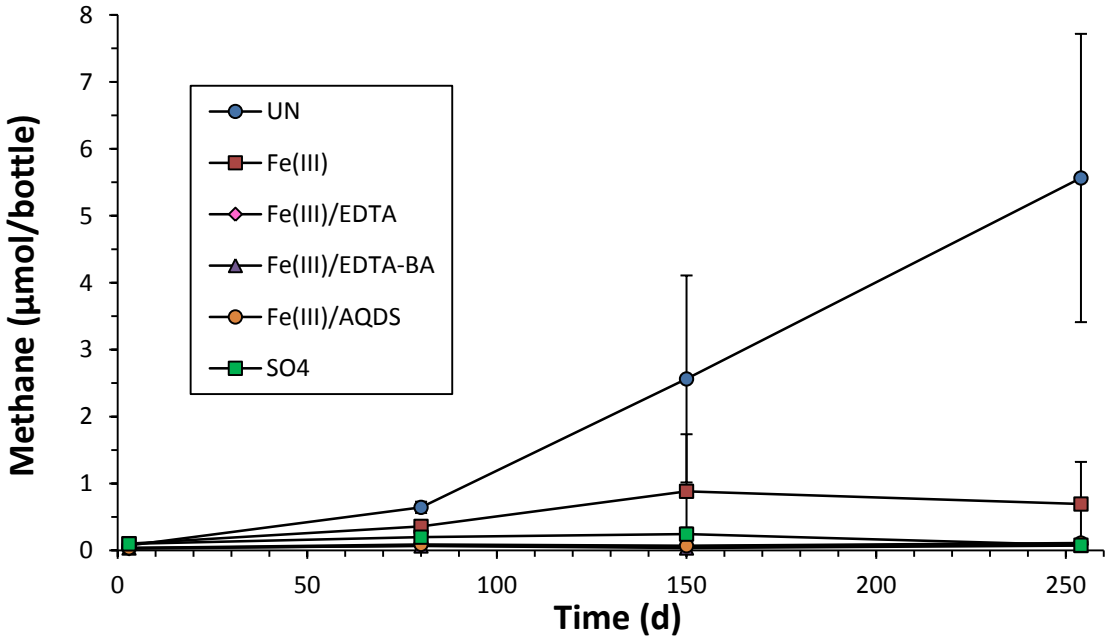

Figure 3.21. Methane results for Site II anaerobic microcosms prepared with soil and groundwater. Data shown are averages for triplicate bottles; error bars represent standard deviations.

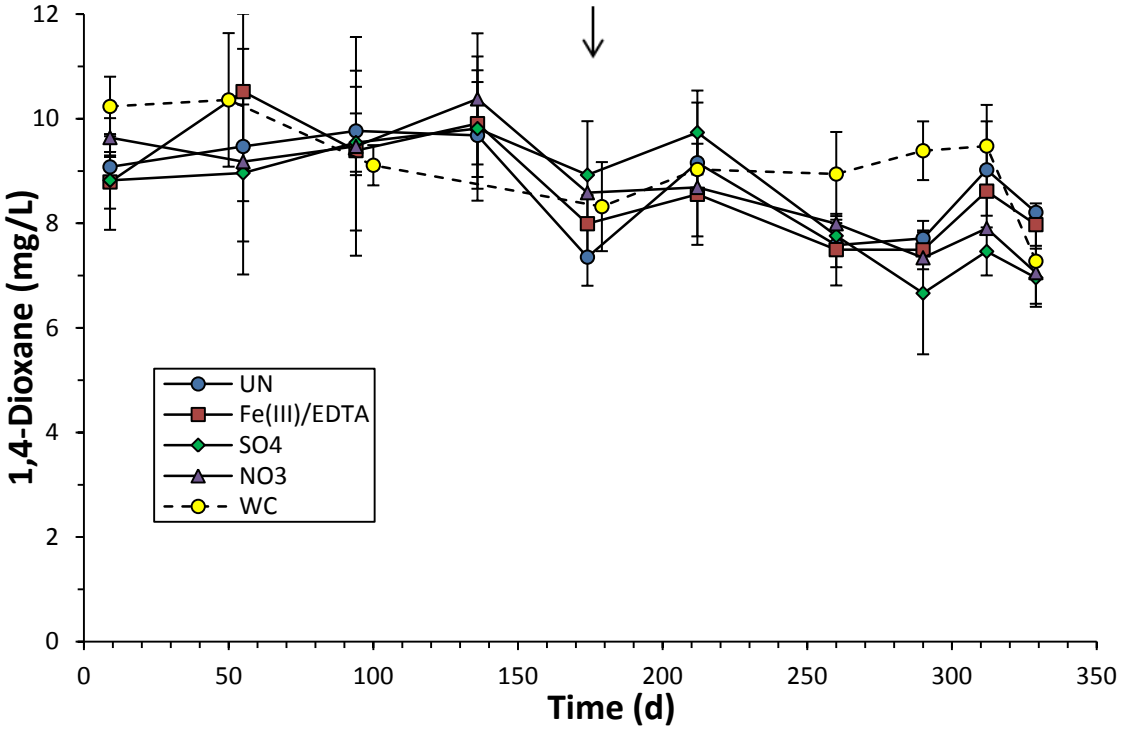

Figure 3.22. 1,4-Dioxane results for the Site II anaerobic microcosms prepared with soil and MSM. Data shown are the averages for triplicate bottles; error bars represent standard deviations. The arrow represents the addition of electron donor to iron and sulfate amended bottles.

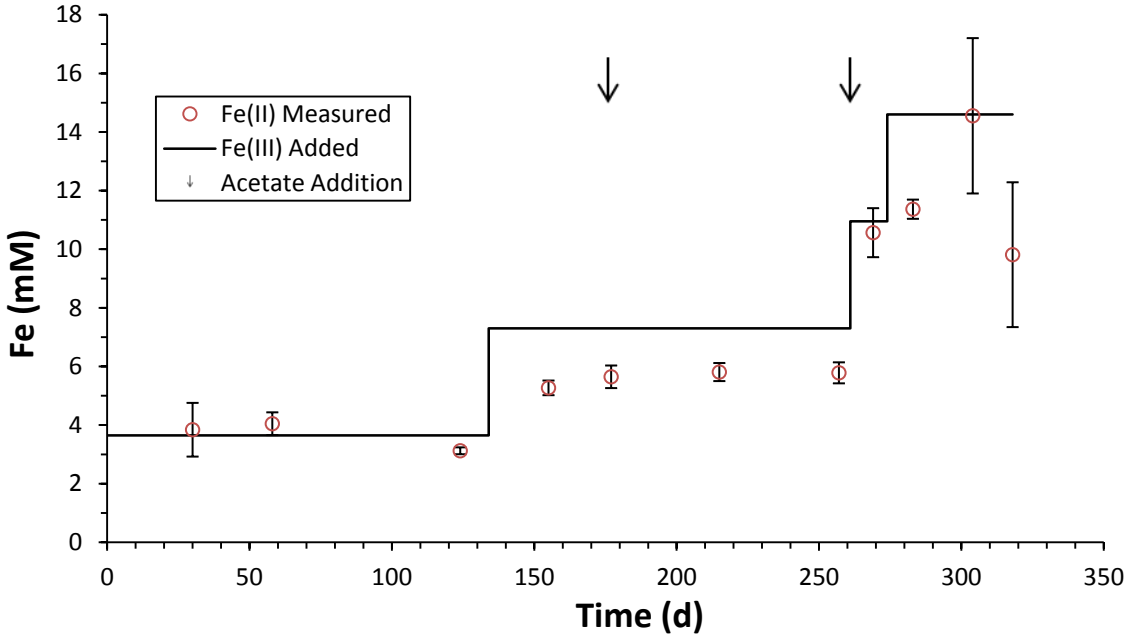

Figure 3.23. Ferrous iron results for Site II anaerobic microcosms prepared with soil and MSM. Data shown are the averages for triplicate bottles; error bars represent standard deviations. The solid line represents the cumulative Fe(III)-EDTA added. The arrow represents addition of acetate.

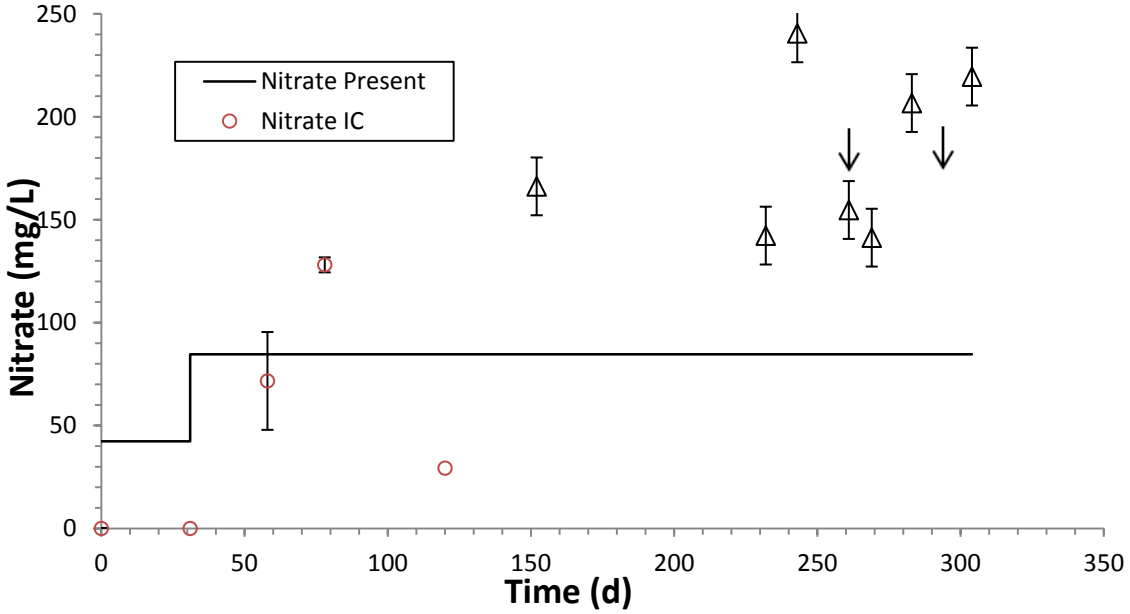

Figure 3.24. Nitrate results for Site II anaerobic microcosms prepared with soil and MSM. Data shown are the averages for triplicate bottles; error bars represent standard deviations. The solid line represents the cumulative concentration of nitrate added.

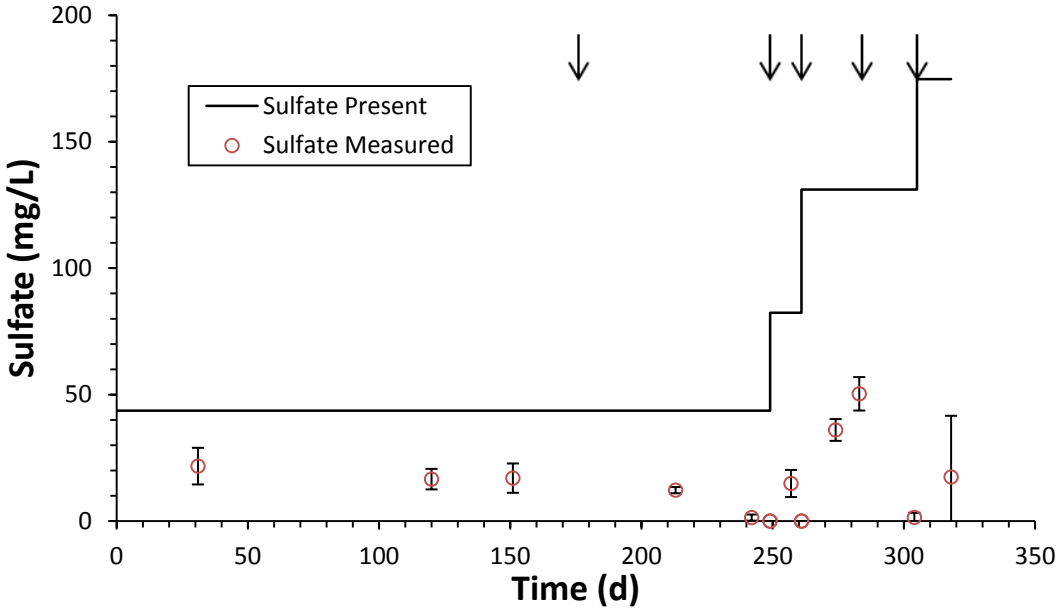

Figure 3.25. Sulfate results for Site II anaerobic microcosms prepared with soil and MSM. Data shown are the averages for triplicate bottles; error bars represent standard deviations. The solid line represents the calculated amount of sulfate initially present plus the amount added. The arrows denote additions of lactate.

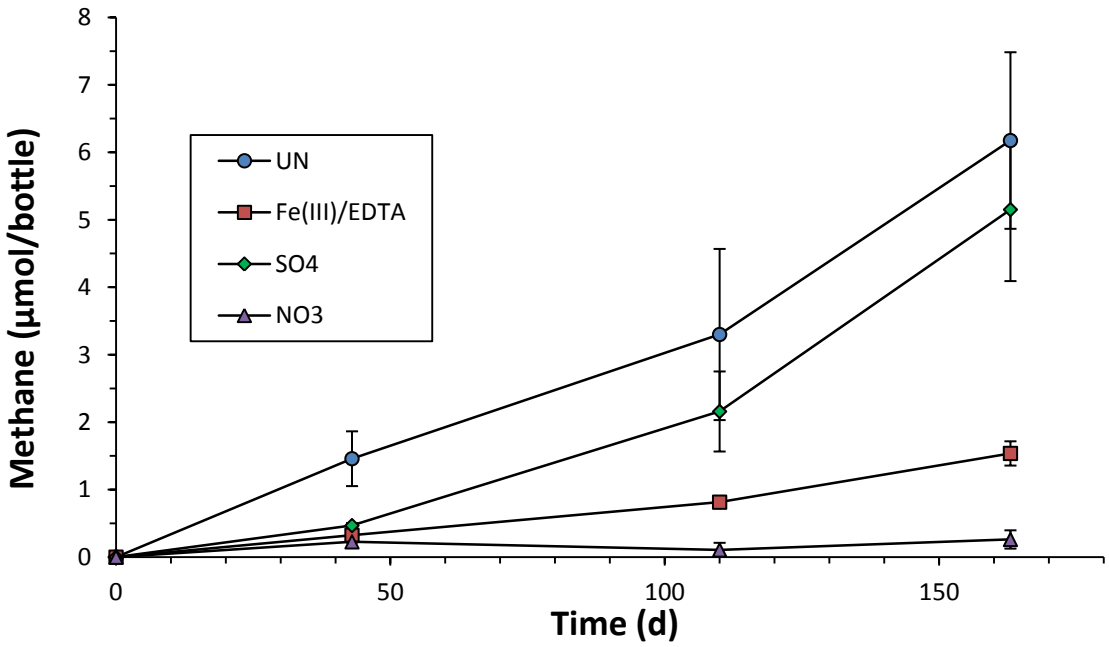

Figure 3.26. Methane results for Site II anaerobic microcosms prepared with soil and MSM. Data shown are averages for triplicate bottles; error bars represent standard deviations.

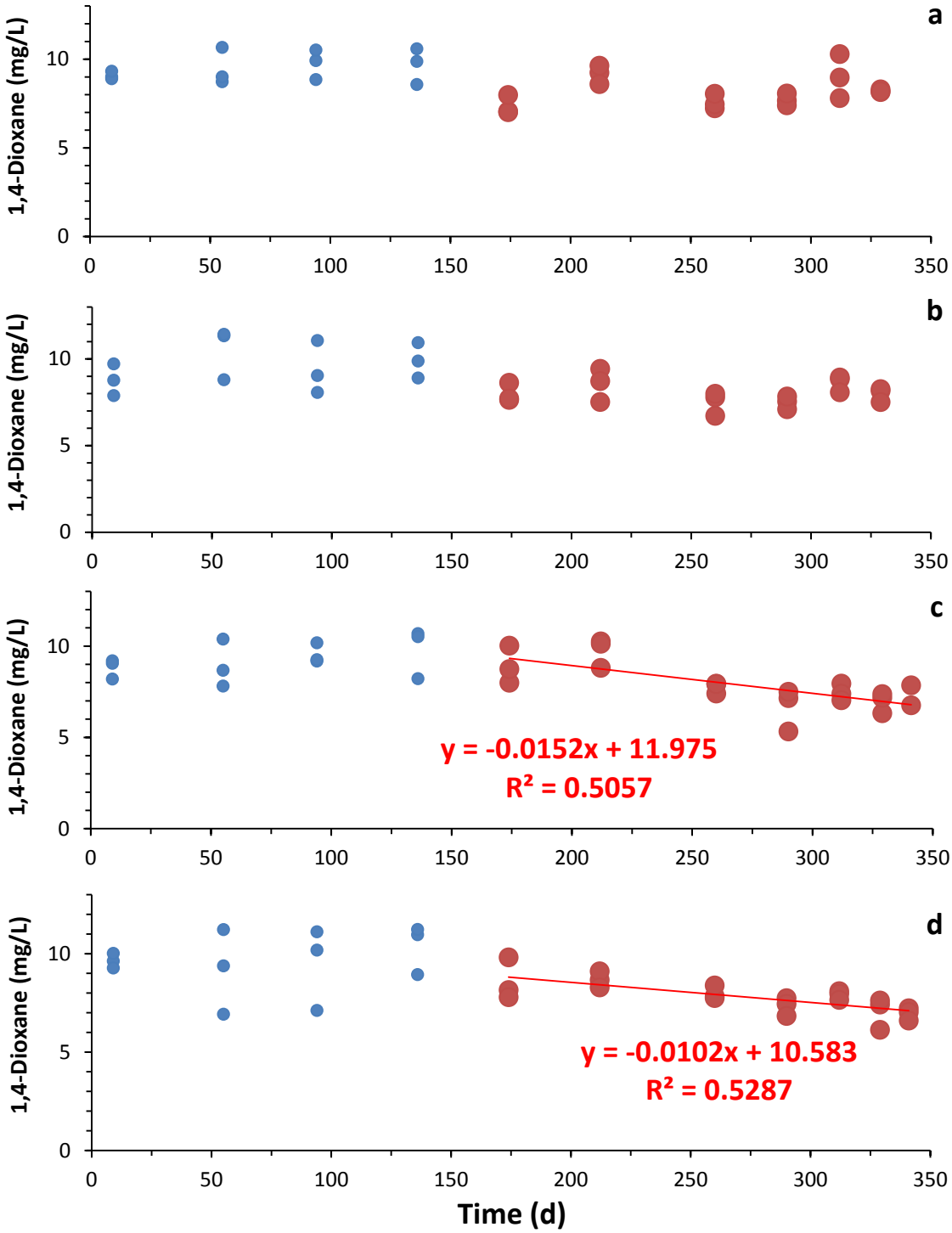

Figure 3.27. Linear regression analysis of Site II MSM microcosms. Statistically significant negative slopes were obtained for the analysis of **c)** sulfate and **d)** nitrate. No significant decrease was obtained for the **a)** unamended and **b)** Fe(III)-EDTA amended microcosms.

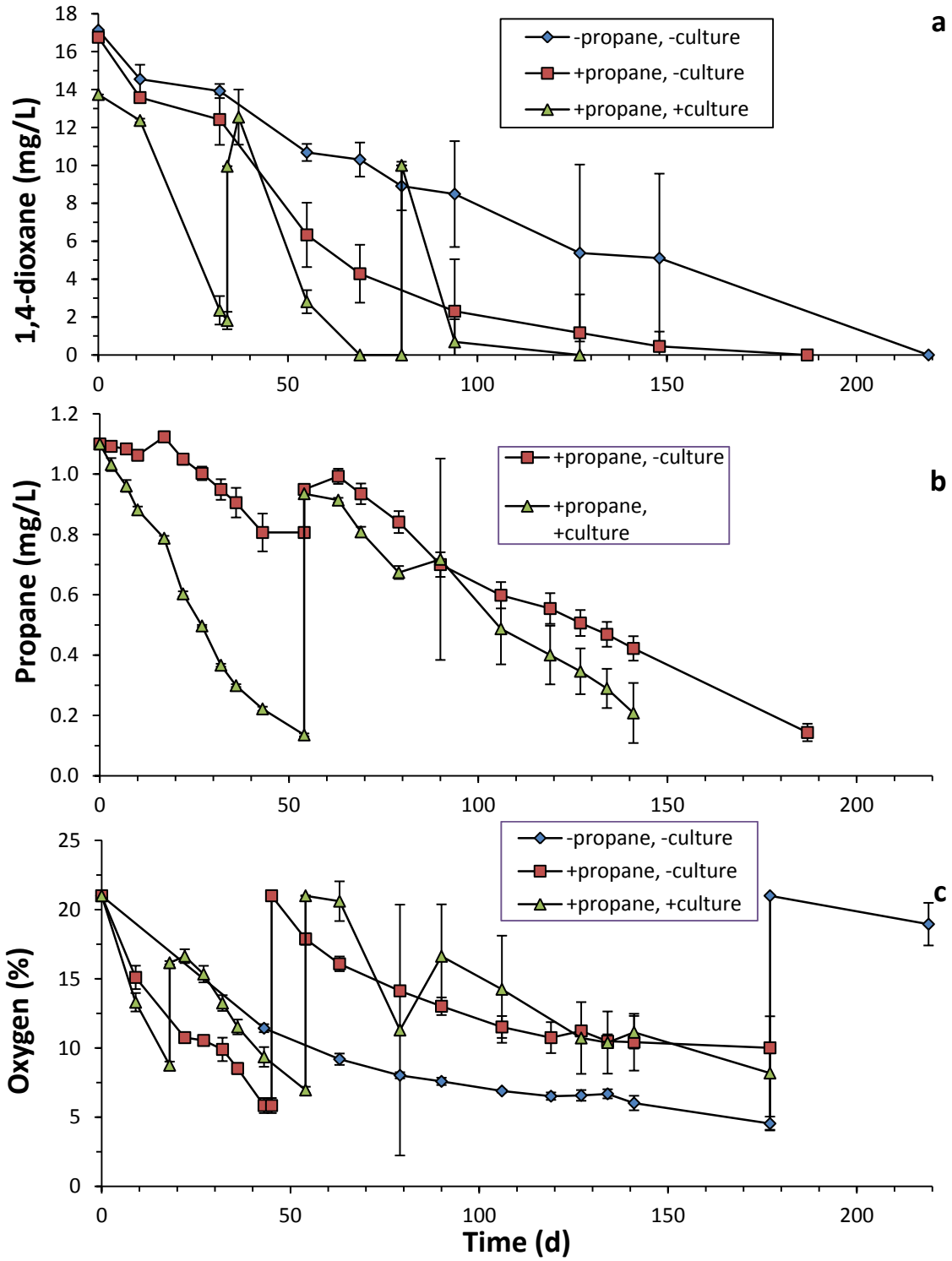

Figure 3.28. Results for Site II aerobic microcosms prepared with soil and groundwater; **a)** 1,4-dioxane; **b)** propane; and **c)** percent oxygen present in microcosm headspace. Data shown are averages for triplicate bottles; error bars represent standard deviations.

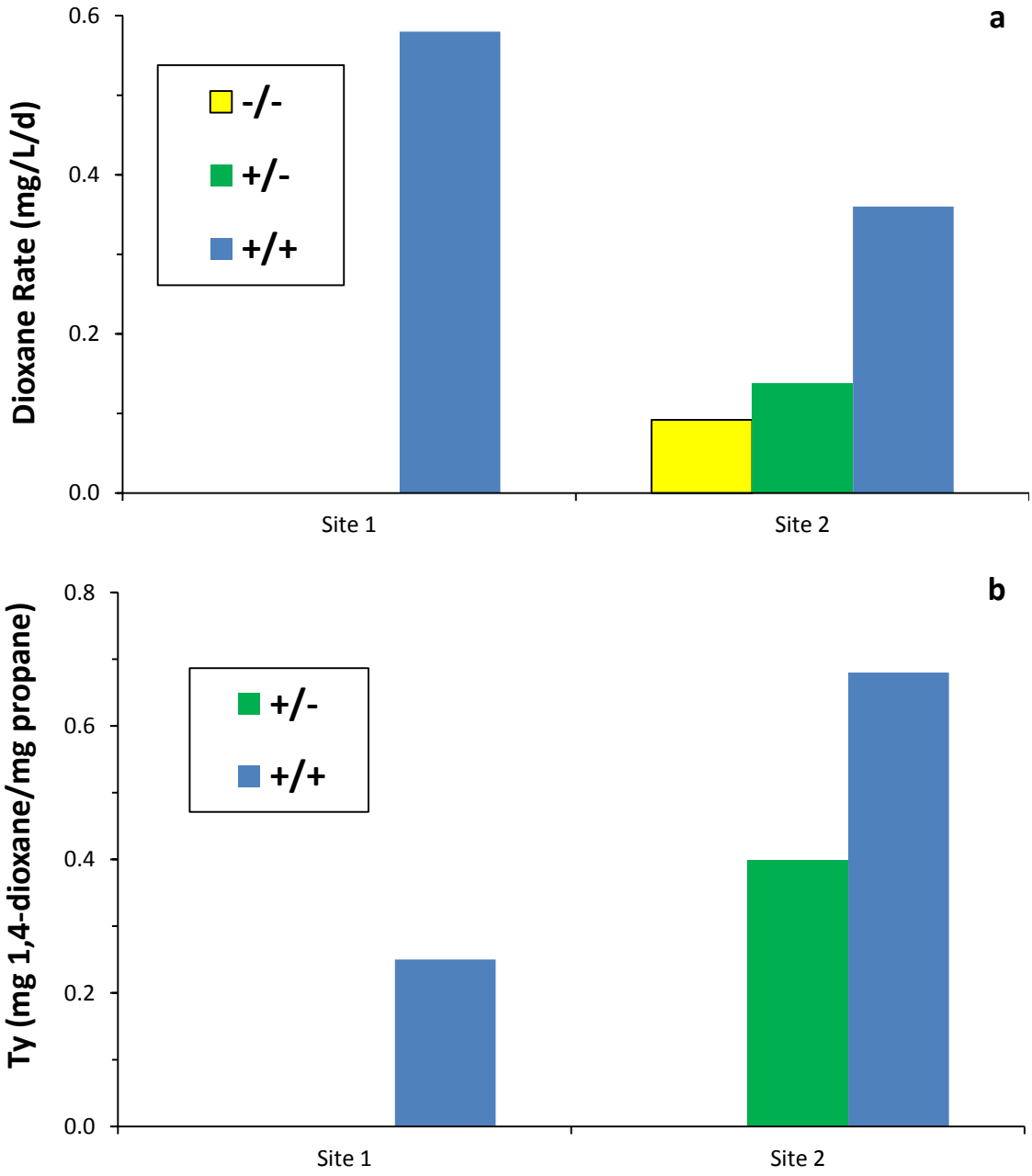

Figure 3.29. Summary of results for the aerobic microcosms prepared with soil and groundwater; **a)** average 1,4-dioxane degradation rates; and **b)** observed transformation yields. The legend indicates the addition (+) or no addition (-) of propane (to the left of the slash) and bioaugmentation (+) or no addition of culture (-) (to the right of the slash).

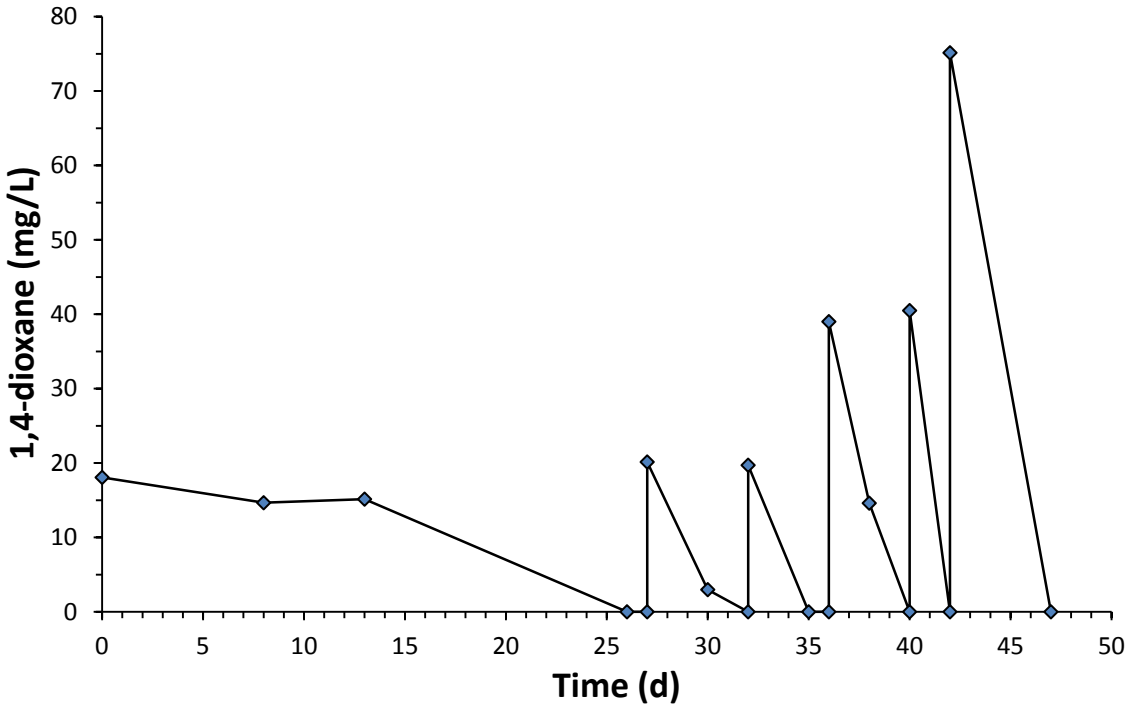

Figure 3.30. Results for enrichment of unamended Site II aerobic microcosms. Data shown is for a single bottle.

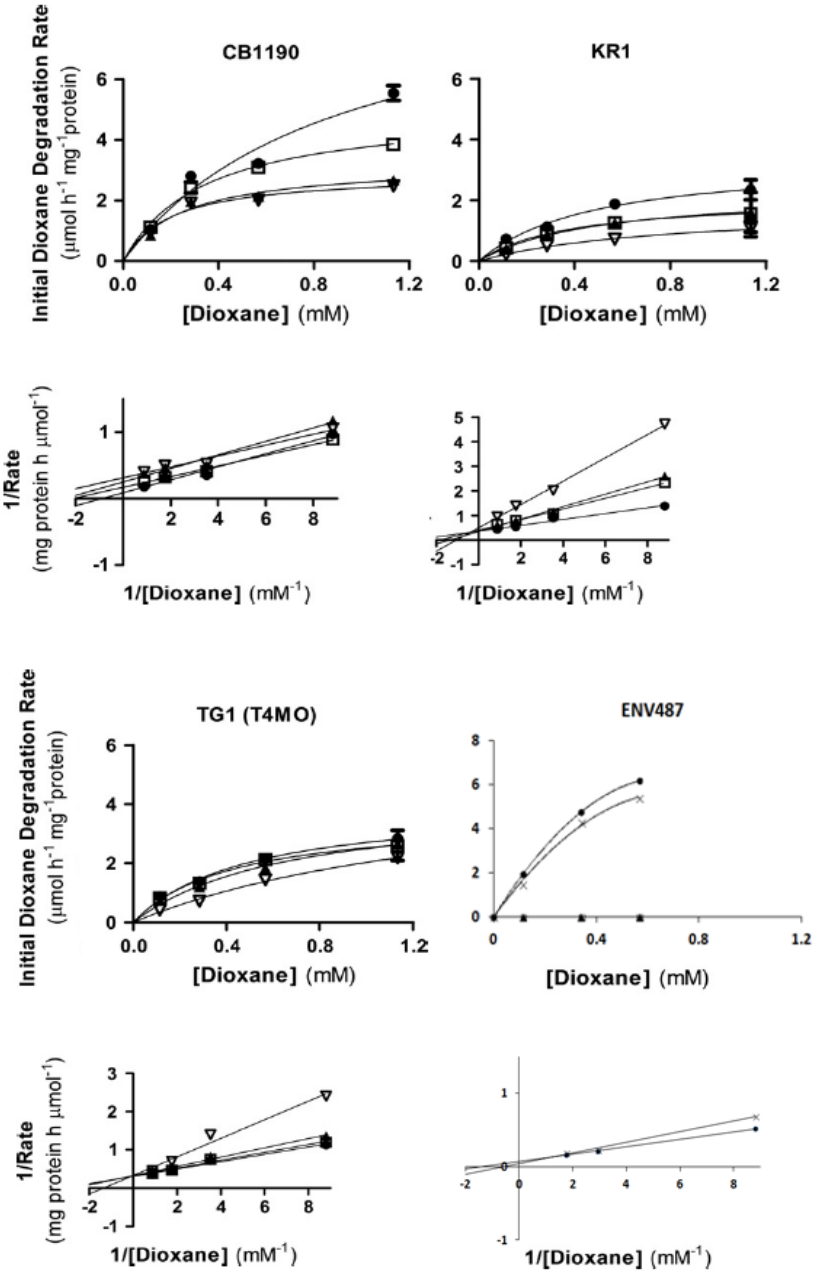

Figure 4.1. Comparison of initial dioxane degradation rates in the presence of varying 1,1-DCE concentrations for ENV487. Data for strains CB1190, KR1, and TG1 reproduced courtesy of Mahendra et al. (2013). Concentration of DCE in each series: closed circles - 0 µM; crosses - 0.1 µM; empty squares - 1 µM; closed triangles - 10 µM; open triangles - 100 µM.

APPENDICES

Appendix A

Protocol to prepare BSM medium

1. Prepare the buffer and metals stock solution according to recipe

2. Stock Solution 20X BSM A buffer, 1 liter:

    Add **85 g** of potassium phosphate dibasic K_2HPO_4-$3H_2O$

    Add **20 g** of sodium phosphate NaH_2PO_4-H_2O

    Add **40 g** of ammonium chloride NH_4Cl

    Add 1 liter of distilled deionized water and stir

    Check pH, the value should be 6.9 for this buffer

3. For stock solution 20X BSM B metals, add

    Nitrillotriacetic acid (NTA) trisodium salt $N(CH_2CO_2Na)_3$-H_2O **2.46 g**

    Magnesium Sulfate $MgSO_4$-$7H_2O$ **4.00 g**

    Ferrous Sulfate $FeSO_4$-$7H_2O$ **0.24 g**

    Manganese Sulfate $MnSO_4$-H_2O **0.06 g**

    Zinc Sulfate $ZnSO_4$-$7H_2O$ **0.06 g**

    Cobalt Chloride $CoCl_2$-$6H_2O$ **0.02 g**

    One liter distilled water: ADJUST WATER TO pH 4-5 with HCl

    PRIOR TO ADDING BSM B CHEMICALS

4. Mix 50 mL of 20X BSM A with 50 mL 20X BSM B and add 0.9 L of distilled deionized water

5. Measure pH, the value should be neutral.

Appendix B

NUTRIENT MEDIA PREPARATION

STEP 1: Prepare 100 mL of a 100X stock of the trace element solution SL9. The stock trace element solution SL9 contains [2]:

Chemical	MW (g/mol)	mg/L*	mg/100 mL
Nitrilotriacetic acid ($N(CH_2CO_2H)_3$	191.14	12,800	1,280
$FeCl_2 \cdot 4H_2O$	198.81	200	20
$CoCl_2 \cdot 6H_2O$	207.01	19,000	1,900
$MnCl_2 \cdot 2H_2O$	161.87	10,000	1,000
$ZnCl_2$	136.32	7,000	700
H_3BO_3	61.83	600	60
$NiCl_2 \cdot 6H_2O$	237.69	2,400	240
$CuCl_2 \cdot 2H_2O$	170.48	200	20
$Na_2MoO_4 \cdot 2H_2O$	241.95	3,600	360

STEP 2: Dilute the stock 100X in DDI water by adding 1 mL of the stock to 99 mL of DDI.

STEP 3: Prepare 100 mL of a 100X stock of the selenite-tungstate solution. The selenite-tungstate solution contains [2]:

Chemical	MW (g/mol)	mg/L	mg/100 mL
NaOH	40.00	50,000	5,000
$Na_2SeO_3 \cdot 5H_2O$	263.01	300	30
$Na_2WO_4 \cdot 2H_2O$	329.85	400	40

Note: $Na_2SeO_3 \cdot 5H_2O$ may not be available in its hydrated form, therefore if we use the dehydrated form (Na_2SeO_3; MW = 172.94 g/mol), therefore, in order to obtain the goal concentration, the next consideration is made:

STEP 4: Dilute the stock 100X in DDI water by adding 1 mL to 99 mL.

STEP 5: Prepare 100 mL of a 10X stock of the vitamin solution. The vitamin solution contains [1]:

Chemical	Molecular Structure	MW (g/mol)	mg/L	mg/100 mL
p-aminobenzoate	$C_7H_6NO_2$	136.13	400	40
Biotin	$C_{10}H_{16}N_2O_3S$	244.31	100	10
Nicotinic acid	$C_6H_5NO_2$	123.11	1,000	100
Pantothenic acid	$C_9H_{17}NO_5$	219.23	500	50
Pyridoxine	$C_8H_{11}NO_3$	169.18	1,500	150
Thiamine	$C_{l2}H_{17}N_4OS$	265.35	1,000	100
Cobalamin	$C_{63}H_{88}CoN_{14}O_{14}P$	1,355.37	1,000	100

Notes:

- ✓ *P*-aminobenzoate may be available as *p*-aminobenzoic acid ($C_7H_7NO_2$; MW = 137.14 g/mol)
- ✓ D-Biotin form is the same as Biotin in terms of content and functions.
- ✓ Pantothenic acid may be available as *p*-pantothenic acid hemicalcium salt ($C_9H_{16}NO_5 \cdot 1/2Ca$; MW = 238.27 g/mol)
- ✓ Thiamine may be available as thiamine hydrochloride ($C_{l2}H_{17}ClN_4OS$; MW = 300.81 g/mol)

STEP 6: Dilute the stock 10X in DDI water by adding 10 mL to 90 mL.

STEP 7: Prepare the basal medium. The basal medium contains [1]:

Chemical	MW (g/mol)	g/L*
NaCl	58.44	1
KH_2PO_4	136.09	0.2
NH_4Cl	53.49	0.27
$MgCl_2 \cdot 6H_2O$	203.30	0.41
KCl	74.55	0.52
$CaCl_2 \cdot 2H_2O$	147.01	0.15

* In grams per liter of deionized water.

STEP 8: Autoclave the basal medium for one hour (method #1 for Liquids on the autoclave downstairs).

STEP 9: Cool it down under 70% N_2/30% CO_2 (achieved by sparging the headspace with a canula) atmosphere to 60°C.

STEP 10: After cooling (but while continuing to sparge with 70% N_2/30% CO_2), add 2.5 g of $NaHCO_3$ into the liter solution.

STEP 11: While continuing to sparge, add 1 mL (0.1% (vol/vol)) per liter of trace element solution SL9, 0.5 mL (0.05% (vol/vol)) of vitamin solution, 1 mL (0.1% (vol/vol)) of selenite-tungstate solution.

STEP 12: While continuing to sparge , check the pH, which should be neutral (around 7.0 to 7.5). If it is necessary adjust it with sterile, anoxic HCl.

STEP 13: While continuing to sparge, add 0.5 mg of resazurin per liter of solution to indicate the solution's redox potential.

STEP 14: While continuing to sparge, rinse the outside of some $Na_2S{\cdot}9H_2O$ (MW = 240.182) crystals with DDI water and pat dry with a Kim wipe. Add 0.48 g (2 mM) of $Na_2S{\cdot}9H_2O$ as the reducing agent.

STEP 15: While removing the canula, cap the bottle and transfer the medium to the glove box.

Appendix C

STANDARD OPERATING PROCEDURE

MATERIALS and/or EQUIPMENT:

- Ammonium Minimal Salt Medium (Per liter): See Chemical Inventory and MSDS located in each lab for more information on use.
    - *Change pH of media (first six items) before autoclaving to pH 7.2-7.3 by using 1 M NaOH or HCl. Buffer solution is added after autoclaving and after media has cooled.
    - $MgSO_4 \cdot 7H_2O$ 1 g
    - $(NH_4)_2SO_4$ 0.66 g
    - $CaCl_2 \cdot 2H_2O$ 0.015 g
    - Stock A 1 mL
        - Stock A (per liter)
            - Fe-Na EDTA 5.0 g
            - $NaMoO_4 \cdot 2H_2O$ 2.0 g
    - Phosphate buffer 20 mL
    - Trace Metals 1 mL
        - Trace Metal Solution (per liter) – store 1 L in fridge
            - $FeSO_4 \cdot 7H_2O$ 0.5 g/L
            - $ZnSO_4 \cdot 7H_2O$ 0.4 g/L
            - $MnSO_4 \cdot H2O$ 0.02 g/L
            - $CoCl_2 \cdot 6H_2O$ 0.05 g/L
            - $NiCl_2 \cdot 6H_2O$ 0.01 g/L
            - H_3BO_3 0.015 g/L
            - $CuCl_2 \cdot 2H_2O$ 0.005 g/L
            - EDTA 0.25 g/L
    - Buffer Solution 10 mL
        - 113 g/L KH_2PO_4 and 47 g/L Na_2HPO_4 in 1000 mL DI water
    - DI Water 980 mL
    - 2 - 8 % v/v 1,4-dioxane

REFERENCES

Adamson, T.D.; Mahendra, S.; Walker, Jr., K.L.; Rauch, S.R.; Sengupta, S.; Newell, C.J., A multisite survey to identify the scale of the 1,4-dioxane problem at contaminated groundwater sites. *Environ. Sci. Technol. Lett.* **2014**, *1*, (5), 254-258.

Adamson, T.D.; Anderson, R.H.; Mahendra, S.; Newell, C.J., Evidence of 1,4-dioxane attenuation at groundwater sites contaminated with chlorinated solvents and 1,4-dioxane. *Environ. Sci. Technol.* **2015**, *49*, 6510-6518.

Adrian, L.; Manz, W.; Szewzyk, U.; Görisch, H., Physiological characterization of a bacterial consortium reductively dechlorinating 1,2,3- and 1,2,4-trichlorobenzene. *Appl. Environ. Microbiol.* **1998,** *64*, (2), 496-503.

Anderson, J. E.; McCarty, P. L., Transformation yields of chlorinated ethenes by a methanotrophic mixed culture expressing particulate methane monooxygenase. *Appl. Environ. Microbiol.* **1997,** *63*, (2), 687-693.

Annweiler, E.; Michaelis, R.; Meckjenstock, R.U., Anaerobic cometabolic conversion of benzothiophene by a sulfate-reducing enrichment culture and in a tar-oil-contaminated aquifer. *Appl. Environ. Microbiol.* **2001**, *3*, (12), 5077-83.

Azman, S.; Khadem, A.F.; Zeeman, G.; van Lier, J.B.; Plugge, C.M., Mitigation of humic acid inhibition in anaerobic digestion of cellulose by addition of various salts. *Bioeng.* **2015**, *2*, (2), 54-65.

Finneran, K.T.; Lovley, D.R., Anaerobic degradation of methyl tert-butyl ether (MTBE) and tert-butyl alcohol (TBA). *Environ. Sci. Technol.* **2001**, *35*, (9), 1785-90.

Hopkins, G. D.; Semprini, L.; McCarty, P. L., Trichloroethylene concentration effects on pilot field-scale in-situ groundwater bioremediation by phenol-oxidizing microorganisms. *Environ. Sci. Technol.* **1993,** *27*, (12), 2542-2547.

Jitnuyanont, P.; Sayavedra-Soto, L.; Semprini, L., Bioaugmentation of butane-utilizing microorganisms to promote cometabolism of 1,1,1-trichloroethane in groundwater microcosms. *Biodegradation* **2001,** *12*, (1), 11-22.

Kim, Y.; Semprini, L.; Arp, D. J., Aerobic cometabolism of chloroform and 1,1,1-trichloroethane by butane-grown microorganisms. *Bioremediation Jour.* **1997,** *1*, (2), 135-148.

Lippincott, D.; Streger, S.H.; Schaefer, C.E.; Hinkle, J.; Stormo, J.; Steffan, R.J., Bioaugmentation and propane biosparging for in situ biodegradation of 1,4-dioxane. *Groundwater Monitoring and Remediation* **2015**, *35* (2), 81-92.

Mahendra, S.; Petzold, C.J.; Baidoo, E.E.; Keasling, J.D.; Alvarez-Cohen, L., Identification of the intermediates of in vivo oxidation of 1,4-dioxane by monooxygenase-containing bacteria. *Environ. Sci. Technol.* **2007**, *41*, (21), 7330-6.

Mahendra, S.; Grostern, A.; Alvarez-Cohen, L., The impact of chlorinated solvent co-contaminants on the biodegradation kinetics of 1,4-dioxane. *Chemosphere* **2013,** *91*, (1), 88-92.

Mohr, T. K.G.; Stickney, J. A.; DiGuiseppi, W.H., Environmental Investigation and Remediation: 1,4-Dioxane and Other Solvent Stabilizers. CRC Press: Boca Raton, Florida, 2010.

Parales, R.E.; Adamus, J.E.; White, N.; May, H.D., Degradation of 1,4-dioxane by an actinomycete in pure culture. *Appl. Environ. Microbiol.* **1994**, *60*, (12), 4527-30.

Rothermich, M. M.; Hayes, L. A.; Lovley, D. R., Anaerobic, sulfate-dependent degradation of polycyclic aromatic hydrocarbons in petroleum-contaminated harbor sediment. *Environ. Sci. Technol.* **2002,** *36*, (22), 4811-4817.

Shen, W.; Chen, H.; Pan, S., Anaerobic biodegradation of 1,4-dioxane by sludge enriched with iron-reducing microorganisms. *Bioresource Technology* **2008,** *99*, (7), 2483-2487.

Steffan, R. J.; McClay, K. R.; Masuda, H.; Zylstra, G. J. *Biodegradation of 1,4-Dioxane*; Shaw Environmental Inc., Final Report to SERDP for Project CU-1422: 2007.

Stookey, L. L., Ferrozine: a new spectrophotometric reagent for iron. *Anal. Chem.* **1970,** *42*, 779-781.

Tschech, A.; Pfennig, N., Growth yield increase linked to caffeate reduction in *Acetobacterium woodii*. *Arch. Microbiol.* **1984,** *137*, (2), 163-167.

United States Environmental Protection Agency (2014) Technical Fact Sheet - 1,4-Dioxane. USEPA. Retrieved from http://www2.epa.gov/sites/production/files/2014-03/documents/ffrro_factsheet_contaminant_14-dioxane_january2014_final.pdf.

Yu, R.; Peethambaram, H.S.; Falta, R.W.; Verce, M.F.; Henderson, J.K.; Bagwell, C.E.; Brigmon, R.L.; Freedman, D.L. Kinetics of 1,2-dichloroethane and 1,2-dibromoethane biodegradation in anaerobic enrichment cultures. *Appl. Environ. Microbiol*. **2013,** *79*, (4), 1359-1367.

Zenker, M. J.; Borden, R. C.; Barlaz, M. A., Modeling cometabolism of cyclic ethers. *Environ. Eng. Sci.* **2002,** *19*, (4), 215-228.